Novel natural products as Modifiers of Multidrug Resistance

Thesis

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Novel Natural Products as Modifiers of Multidrug Resistance

by
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A thesis submitted in part requirement for the degree of Doctor of Philosophy from the Open University

November 1995

Date of award 5th February 1996

Acknowledgements

I am grateful to my supervisor, Dr Peter Twentyman, for giving me the opportunity to study in Cambridge and for providing me with such expert supervision and guidance throughout my studies.

My thanks also go to Xenova Ltd for providing me with the funding for my studies. I am especially grateful to Dr Michael Moore, Dr Ian Dale, Dr Hamish Ryder (employees of Xenova Ltd) and Mr Mike Luscombe (formerly an employee of Xenova Ltd), for their help and advice over the past 3 years.

There are many colleagues (past and present) at the Clinical Oncology and Radiotherapeutics Unit who have offered me their help, advice and friendship. In particular: Dr Carolien Versantvoort for help with the phosphorylation assay, Chapter 7; Mrs Tanya Bagrij for help with the GSH assays, Chapter 6; Mrs Pauline Finbow for help (and patience) with word processing and Dr Julie Reeve for her general help and encouragement. Special thanks go to Karen Wright not only for her help in the laboratory but for her continued support and friendship over the past 3 years.

I thank the awards committee of the British Association for Cancer Research for awarding me the Gillard fellowship. This funding enabled me to spend time in the laboratory of Dr M Center (Kansas State University, Manhattan, Kansas) and Dr S Bates (NCI, Bethesda, Maryland) to learn the phosphorylation assay.

Both of my parents have, as always, been a great source of support and encouragement.

Finally, I thank my fiance, Jason, for his commitment and patience over the past 3 years especially during the writing of this thesis. Not only has he been my cook, cleaner, comforter and proof reader but he has kept me cheerful (most of the time) through those long days (and nights) of sitting in front of a computer screen writing this thesis.

Abstract

Novel Natural Products as Modifiers of Multidrug Resistance

Deoxyspergualin, an immunosuppressive antibiotic, is similar to cyclosporin A and FK506 in some of its properties and mechanisms of action. We therefore decided to examine the effects of deoxyspergualin in multidrug resistant (MDR) cells. Deoxyspergualin did not alter the drug accumulation deficit observed in the MDR, P-glycoprotein-overexpressing cell lines EMT6/AR1.0 or H69/LX4. In addition it was not able to inhibit the ability of [^{3}H]azidopine to photolabel P-glycoprotein. It is unlikely, therefore, that deoxyspergualin is a substrate for P-glycoprotein. Our data suggests that deoxyspergualin is not a good candidate for development as a resistance modifier. Its maintenance of activity in classical MDR cells and its potent *in vivo* antitumour activity, however, makes deoxyspergualin a promising agent for further investigation into its potential clinical use as an antitumour agent.

Several novel fungal products, supplied by Xenova Ltd, were tested for their ability to modify both P-glycoprotein- and MRP-mediated MDR. We demonstrated that, within this series, the most potent modifiers of MDR contained within their structure a tetrahydroisoquinoline group attached to a benzylidene ring in either the meta or para position together with the presence of a diketopiperazine ring. The ability of a molecule to be positively charged and lipophilic at physiological pH are also important determinants of activity as a modifier of P-glycoprotein-mediated MDR. The active compounds inhibit the ability of [3H]azidopine to photolabel P-glycoprotein indicating that they may reverse MDR by themselves binding to P-glycoprotein. Two compounds, XR9006 and XR9051 are effective modifiers of P-glycoprotein-mediated MDR. XR9051 is currently undergoing *in vivo* studies at Xenova.

We discovered a novel fungal product modifier of MRP-mediated MDR, XR9173. This compound, synthesised by scientists at Xenova, is cationic at physiological pH and possesses a tetrahydroisoquinoline group. It is also, therefore, an effective modifier of P-glycoprotein-mediated MDR. Recent findings suggest that MRP is identical to the glutathione (GSH) conjugate transporter (Jedlitschky *et al.*, 1994). It is unlikely, however, that XR9173 reverses MRP-mediated MDR by competing for the same efflux mechanism as GSH as it is not anionic at physiological pH. Our results provide evidence to support the theory that MRP may co-exist with another transporter which is able to transport cationic drugs across the cell membrane and that these transporters are able , in some way, to regulate each others' activity.

A potential regulatory mechanism for P-glycoprotein function is modification of phosphorylation. We initially intended to examine the effect of the Xenova compounds on P-glycoprotein phosphorylation. Scientists at Xenova demonstrated that the compounds in this series were not substrates for PKC. It is unlikely, therefore, that they would have any effect on the phosphorylation status of P-glycoprotein. We had already started to examine the effect of several inhibitors of PKC and the PKC activator TPA on the phosphorylation of P-glycoprotein and on drug transport. The results of these initial studies proved interesting and so we decided to continue with this study. We demonstrated that the PKC inhibitor calphostin C inhibits the phosphorylation of P-glycoprotein and increases drug accumulation in MDR cells. This indicates that altering P-glycoprotein phosphorylation may indeed alter its function. Interestingly, non-light-activated calphostin C, which does not inhibit PKC, also significantly increased drug accumulation in MDR cells. It appears that other actions of PKC inhibitors with respect to P-glycoprotein, i.e. their ability to bind directly to P-glycoprotein, must also be considered when attempting to correlate effects on P-glycoprotein phosphorylation with effects on drug transport.

Abbreviations

ABC	ATP-binding cassette
ADR/DOX	doxorubicin
ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
ATP	adenosine triphosphate
AUC	area under the curve
BSO	D,L-buthionine S,R-sulfoximine
CHO	Chinese hamster ovary
CLL	chronic lymphocytic leukaemia
DHFR	dihydrofolate reductase
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dTMP	deoxythymidinemonophosphate
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
dUMP	deoxyuridinemonophosphate
EDTA	ethylenediaminetetraacetic acid
GI	gastrointestinal
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione-*S*-transferase
H7	1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine
IC_{50}	50% growth inhibitory concentration
kDa	kilodalton
LRP	lung resistance-related protein
MDR	multidrug resistance
mRNA	messenger RNA
MRP	multidrug resistance-associated protein
NSCLC	non-small cell lung cancer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PLL	prolymphocytic leukaemia
RNA	ribonucleic acid
RT-PCR	reverse transcription-PCR

SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
TPA	12-0-Tetradecanoylphorbol 13-acetate
XR	Xenova

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1.1 Introduction to cancer chemotherapy

1.1.1 The History of cancer chemotherapy

The earliest documents known to mention the occurrence and treatment of cancer are medical manuscripts of Chinese and Sumerian origin, from the third millennium BC. Physicians thought then that cancer was caused by the derangement of regulatory processes and tried to correct this with the use of acupuncture. Hippocrates (460-377 BC.) is considered to be the founder of scientific medicine as practised in the West. His works contain numerous references to the causes and treatment of cancer. In his writings malignant tumours are referred to for the first time as carcinomas. He argued that, 'separation of the humours' (blood, phlegm and bile) through toxins arising in the body, particularly 'black bile' caused the disease. He recommended the detoxification of the body using agents such as arsenic, sulphur, laxatives and hellebore. Surgical intervention, he wrote, should be used as a last resort. Clarus Galen (131-200 AD.), the founder of experimental physiology and pathology, wrote five hundred works, including various manuscripts on cancerology. He considered 'black bile', *atra bilis*, of particular importance, regarding it as the cause of cancer. This dogma remained, undisputed, into the eighteenth century.

'The Complete Housewife or Accomplished Gentlewoman's Companion', published in 1729, suggests the following cures for cancer:

1. An approved remedy for cancer of the breast: Take the hard knobs or warts which grow on the legs of a Stone-Horse, dry them carefully and powder them; give from one scruple to half a dram, every morning and evening in a glass of sack. You must continue taking them for a month or six weeks, or longer if the cancer is far gone.

2. For a cancer in the mouth; take celandine, columbine, sage and fennel, of each one handful, stamp and strain them and to the juices put a spoonful of honey, half a spoonful of burnt alum: mix and beat all these together and wrap in a little flax (about a stick) and rub the cancer with it: if it bleeds 'tis the better.

3. To disperse tumours; take half a pound of figs, two ounces of White Lilly-root, two ounces of bean flour or meal; boil these in water 'til it comes to a poultice; spread it thick on a cloth, apply it warm and swift as often as it goes dry.

(Information taken from: Issels, J., Cancer a Second Opinion: Published 1975)

Fortunately, in the last 250 years cancer treatments have developed greatly from these early herbal remedies. However, the first documented demonstration that a chemical agent was capable of causing remission in a human malignancy was not until 1942, when a patient with lymphoma obtained a brief clinical remission after treatment with the alkylating agent nitrogen mustard (Gilman, 1963). Following this initial documentation, a large number of drugs have been developed and tested for their potential clinical activity. Table 1.1 shows the historical development of chemotherapy.

Table 1.1

The historical development of cancer chemotherapy

DATE	AGENT	DISEASE TREATED
1865	Potassium Arsenite	Leukaemias, various malignancies
1893	Coley's Toxins	Various malignancies
1941	Oestrogens Androgens	Carcinoma of the prostrate/breast
1942	Nitrogen Mustard	Lymphomas, solid tumours
1948-1950	Adrenocorticoids Antifolates (Methotrexate)	Leukaemias, lymphomas, multiple myeloma Acute leukaemia, choriocarcinoma
1950-1955	Busulfan 6-mercaptopurine Actinomycin D Chlorambucil	Chronic granulocytic leukaemia Acute leukaemia Wilms tumour, testicular tumour, choriocarcinoma CLL, non-Hodgin's lymphoma, Hodgkin's disease, ovarian cancer
1955-1960	5-Fluorouracil Cyclophosphamide *Vinca* alkaloids Mitomycin D	Carcinoma of the breast/GI tract Lymphomas, solid tumours Lymphomas, acute leukaemias GI tumours
1960-1965	Hydroxyurea Mithramycin Nitrosoureas Daunorubicin	Chronic granulocytic leukaemia Testicular cancer Lymphomas, brain tumours Acute leukaemia
1965-1970	L-Asparaginase Dacarbazine(DTIC) 6-Thioguanine Cytosine Arabinoside	Acute leukaemia Melanoma Acute leukaemia Acute myeloblastic leukaemia
1970-1975	Doxorubicin Bleomycin	Various tumours Lymphomas, head/neck cancer
1975-1980	Antioestrogens Cisplatin	Breast cancer Testicular, lung and ovarian cancer
1980-1985	Etoposide(VP-16) Mitoxantrone	Small cell lung cancer Breast cancer
1985-1990	Interferon Carboplatin Ifosfamide/mesna	Leukaemias As cisplatin As cyclophosphamide
1990-present	Taxol Fludarabine	Ovarian cancer Leukaemias

1.1.2 Classification and pharmacology of chemotherapeutic agents

Anticancer agents may be classified into three main groups according to their biochemical activities or their origins (Table 1.2). These groups include the alkylating agents, antimetabolites and natural products that have anticancer activity. Inevitably, some drugs appear in more than one class.

Table 1.2

Major families of drugs used in cancer chemotherapy

ALKYLATING AGENTS	ANTIMETABOLITES	NATURAL PRODUCTS	MISC.
Nitrogen Mustards Chlorambucil Melphalan Cyclophosphamide Busulfan Nitrosoureas: (e.g. BCNU, CCNU & Methyl-CCNU) Mitomycin C	Methotrexate 5-Fluorouracil Cytosine Arabinoside 6-Thioguanine 6-Mercaptopurine	Doxorubicin Daunorubicin Actinomycin C Bleomycin Vinblastine Vincristine VP-16 (Etoposide) Taxol® Mitomycin C	Cisplatin DTIC (Dacarbazine) Procarbazine

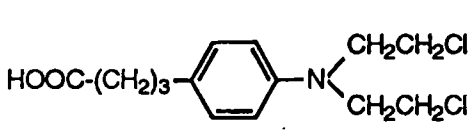
(i) Alkylating agents

Alkylating agents, although chemically diverse in structure, are all able to undergo transformation to produce electrophilic (electron deficient) reactive intermediates. These reactive groups are capable of forming covalent bonds on nucleophilic (excess electrons) moieties on biological molecules including amino, phosphate, sulphydryl and hydroxyl groups. The process of covalent binding to alkyl groups is referred to as alkylation. Alkylating agents may be mono- or bifunctional depending on the presence of either one or two reactive groups on the molecule. Nucleophilic chemical groups that are potential sites of alkylation occur on almost all biological molecules,

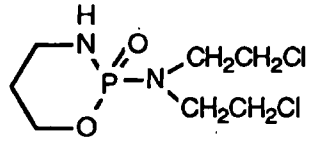
alkylation of bases in DNA appears to be the major cause of lethal toxicity (Figure 1.1.b). Bifunctional alkylating agents (the most common form of alkylating agent used in the clinic) cause cross linking of DNA strands, whereas the toxicity of monofunctional alkylating agents may be related to single strand breaks in DNA or damaged bases. Figure 1.1.a shows the structure of some alkylating agents commonly used in the clinic and Figure 1.1.b shows the formation of a reactive intermediate followed by alkylation at the N-7 position of the DNA base, guanine by nitrogen mustard.

Figure 1.1

(a) Structure of alkylating agents commonly used in the clinic

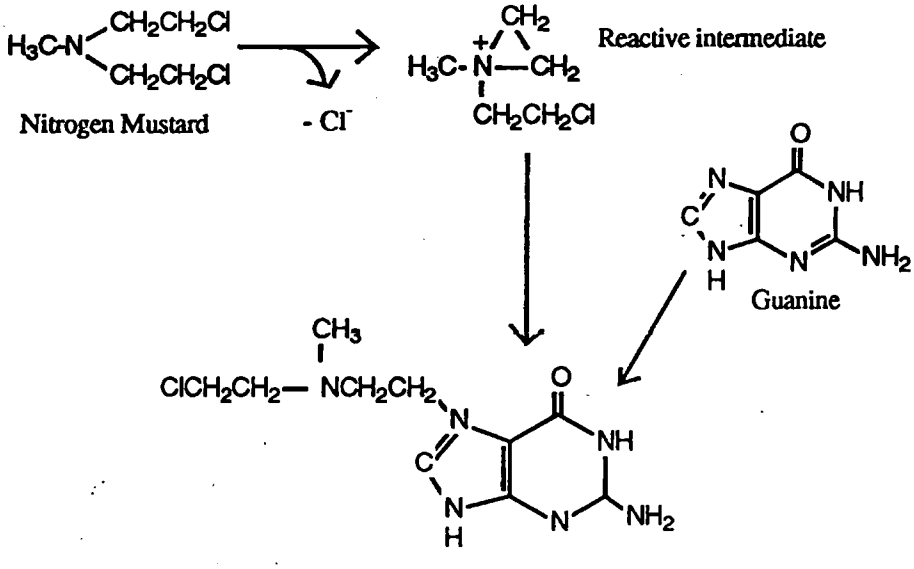


Chlorambucil



Cyclophosphamide

(b) Alkylation at the N-7 position of guanine by nitrogen mustard



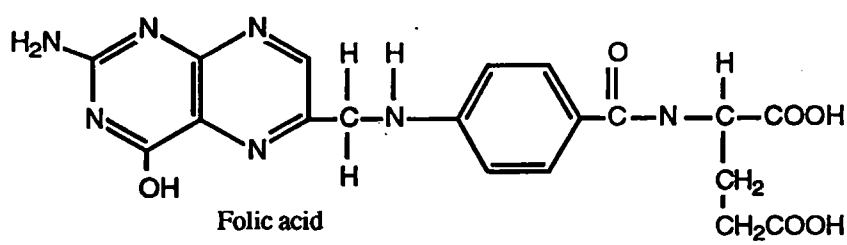
Guanine, alkylated at N-7

(ii) Antimetabolites

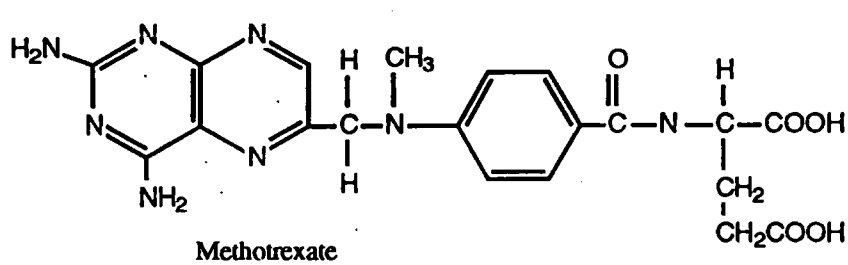
Antimetabolites are drugs which have been synthesised to inhibit critical biochemical pathways, usually leading to inhibition of DNA and RNA synthesis, and tend to be cell cycle dependent. For example, reduced folate is required for transfer of methyl groups in the biosynthesis of purines and in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a reaction which is catalysed by thymidilate synthetase. Reduced folate becomes oxidised in the latter reaction, and its regeneration is dependent on the enzyme dihydrofolate reductase (DHFR) for further reduction to the active form. Methotrexate is a competitive inhibitor of DHFR and thus prevents the formation of reduced folate. This inhibition may lead to a decrease in the availability of dTMP and/or purines leading to a cessation of DNA synthesis and ultimately cell death. Figure 1.2 shows the structure of folic acid, and its analogue methotrexate.

Figure 1.2

Structure of folic acid and methotrexate



Folic acid



Methotrexate

(iii) Natural Products

Many natural product cytotoxic drugs have been derived from products of bacteria, fungi and plants. Table 1.3 illustrates some of these drugs, their source, use, mechanisms of action and toxicities.

Table 1.3

Natural product cytotoxic drugs

DRUG: USE AND TOXICITY	MECHANISM OF ACTION
Doxorubicin (*Streptomyces*) USE: solid tumours **Daunorubicin** USE: leukaemias TOXICITY: cardiotoxicity, myelosuppression, hair loss, nausea and vomiting, tissue necrosis at injection site and mucosistis	-intercalates between base pairs perpendicular to the long axis of the double helix, leading to unwinding of the DNA helix -causes single and double strand breaks in DNA (DNA Topoisomerase II strand cleavage) -inhibits DNA and RNA synthesis, preferential toxicity for cells in S phase -production of the superoxide radical (0_2^-) leading to oxidative damage -binds to cell membranes
Actinomycin D (*Streptomyces*) USE: childhood tumours TOXICITY: Myelosuppression, GI toxicity and hair loss	-binds to guanosine residues of DNA, planar rings intercalate between adjacent base pairs of the DNA helix. This causes inhibition of the transcription of RNA
Bleomycin (fungal) Active component is bleomycin A2 USE: (in combination) testicular cancer and lymphomas, minor use in cancers of head and neck TOXICITY: interstitial fibrosis of lung, fever, chills, damage to skin	-formation of bleomycin-ferrous iron complex followed by intercalation of the complex, helix unwinding, DNA strand breaks -formation of superoxide/hydroxyl radicals catalysed by bleomycin-ferrous iron complex -preferential toxicity for cells in G2 phase
Vinca Alkaloids(periwinkle plant) USE: (i) **vinblastine**(in combination)-testicular cancer. (ii) **vincristine**-childhood leukaemia. (iii) **both**-lymphomas and solid tumours TOXICITY:vinblastine-myelosuppression, vincristine-neurotoxicity	-binds to tubulin, inhibit its polymeristaion and the formation of microtubules

Table 1.3

Natural product cytotoxic drugs (continued)

DRUG: USE AND TOXICITY	MECHANISM OF ACTION
Podophyllotoxin -VP-16 (Etoposide) (mandrake plant) USE: small cell lung cancer and lymphomas TOXICITY: myelosuppression, hair loss	-DNA Topoisomerase II strand cleavage -binds to tubulin, inhibit its polymerisation but does not effect the formation of microtubules -inhibits nucleoside transport into cells and incorporation into RNA and DNA, causing single strand breaks Maximum activity in late S and early G2 phase
Taxol®(paclitaxel)-Pacific Yew tree (*Taxus brecifolia*) **Taxotere**®(docetaxel)-needles of European Yew tree (*Taxus baccata*) USE:Experimental-Non small cell lung cancer TOXICITY: fluid retention	-promotes assembly of microtubules. Renders them resistant to depolymerisation

1.2 - Factors affecting cell survival after drug treatment

1.2.1 Introduction

Both tumours and normal tissue contain cells that are heterogeneous with respect to proliferative state, intrinsic drug resistance, ability to repair drug-induced damage and cell cycle phase. Each of these factors may influence cell survival after treatment with specific cytotoxic drugs. Additionally, limited diffusion of some drugs may lead to varying dosage to cells within the population. It is important to note that factors affecting survival of cells in tumours in the clinic are far more complicated than those illustrated in this section. The pharmacokinetics and pharmacodynamics of the drug in relation to the body as a whole must also be considered.

1.2.2 Cell proliferation rate

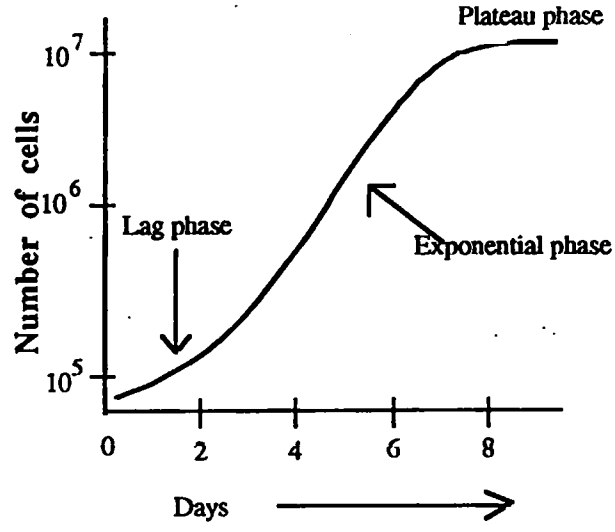
Cell proliferation rate is a major determinant of drug activity. This can be demonstrated in cells grown in culture. Cells in culture grow in a very characteristic fashion. Figure 1.3.a illustrates a typical sigmoid growth curve for cells growing in culture. During 'exponential phase' the cells are cycling rapidly. As cell numbers increase, cell crowding occurs, media nutrients are exhausted and proliferation rate slows due to an increase in cell cycling time and a decrease in growth fraction. Cell death rate increases to equal the rate of cell production and the cells eventually reach 'plateau phase'. Figure 1.3.b shows the difference in the response of rapidly proliferating cells in exponential phase and slowly proliferating cells in plateau phase to exposure to doxorubicin. Exponential phase cells are more sensitive to most chemotherapeutic agents than plateau phase cells (Twentyman *et al.*, 1975). The spleen-colony assay has been used to compare the effects of drugs on cell survival *in vivo* (Bruce *et al.*, 1969; Van Putten *et al.*, 1972). Results from *in vivo* experiments are generally in agreement with those from experiments carried out on cells in culture. Cell proliferation rate therefore appears to influence the ability of cells to survive treatment with cytotoxic drugs.

1.2.3 Cell cycle phase specificity

Almost all drugs show variations in lethal toxicity around the cell cycle (Mauro and Madoc Jones, 1970). For example, many antimetabolites exert lethal toxicity only for cells that are synthesising DNA (i.e. S-phase cells). In contrast, doxorubicin has maximum toxicity for S-phase cells, but also has some activity during other phases. The *vinca* alkaloids, vinblastine and vincristine, disrupt formation of the mitotic spindle. They also cause maximum toxicity in S-phase cells, when formation of the mitotic spindle is initiated. Alkylating agents generally have maximum lethal toxicity in two areas, at the G_2/M and the G_1/S phase boundaries. In the clinic, specificity of many chemotherapeutic agents for certain phases of the cell cycle means that a partly synchronised cell population will remain. It seems, therefore, that scheduling anticancer drug regimens by giving subsequent treatment when a large number of survivors are again in the sensitive phase would maximise total cell kill. *In vivo*, cell cycling times are widespread throughout the cell population and synchrony is lost very rapidly making this type of dosing regimen unrealistic.

Figure 1.3

(a) Typical growth curve for cells in culture



(b) Survival curves for the treatment of exponential and plateau phase cells with an anthracycline (e.g. doxorubicin)

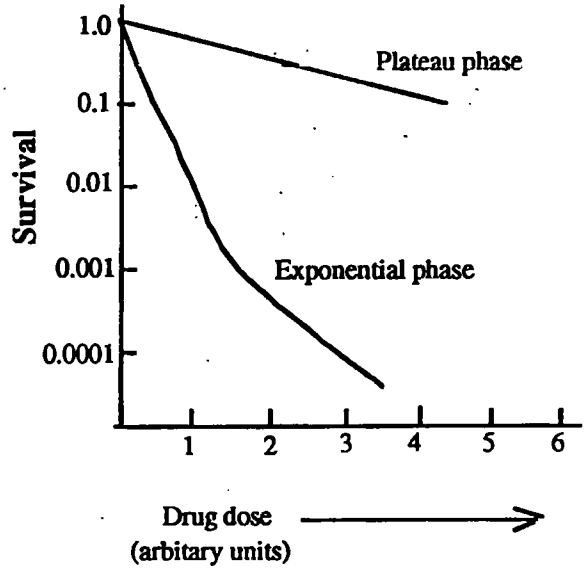


Figure 1.3. (a) A typical sigmoid growth curve for cells in culture. During exponential phase there is rapid cell cycling, but there is minimal cell proliferation during plateau phase. (b) Doxorubicin survival curves for exponential and plateau phase cells (Twentyman *et al.*, 1975) Adapted from Tannock and Hill, 1992.

1.2.4 Drug resistance

The differential sensitivity of chemotherapeutic agents is also dependent on the presence or development of drug resistance. Clinical drug resistance can be divided into two categories. Firstly, many tumour types that occur commonly in man (eg, colon cancer, lung cancer other than the small cell type) are unresponsive to chemotherapy from the initial diagnosis and are therefore said to demonstrate *inherent* resistance. Secondly, other tumour types such as small cell lung cancer, acute myeloid leukaemia and ovarian cancer frequently show a very good response to initial chemotherapy (complete response rate is typically 50%) but almost always the disease recurs within months and displays a progressive resistance to further drug treatment. Such tumours, therefore demonstrate *acquired* resistance. Clinical evidence of acquired drug resistance was observed as early as the 1940s. Good response rates were obtained in adult leukaemias and lymphomas, using nitrogen mustards or anti-folates as single agent therapies. These responses were, however, often short in duration and relapse was associated with acquired drug resistance to the original cytotoxic (Chabner *et al.*, 1990). Acquired resistance may result from the selection for and repopulation of the tumour by intrinsically drug resistant cells as a result of the failure of the first line treatment to eradicate this population. Alternatively, it may result from the induction, by the initial chemotherapeutic agent, of biochemical or genetic alterations which enhance cellular resistance levels to that agent and possible cross resistance to others. Tumours are populated by a heterogeneous mass of cells with differing biochemical, morphological and drug response characteristics. This heterogeneity within the tumour cell population may result from genetic lesions (point mutations or amplification) or changes in the regulation of genes encoding target proteins (Chabner *et al.*, 1990).

It is clear that development of drug resistance is not unique to cancer cells. A wide variety of organisms including bacteria, protozoa and insects are able to develop resistance to a number of toxic agents. Two common examples are the resistance

developed by the malaria parasite, *Plasmodium falciparum*, to various antimalarials including chloroquine and proguanil and the bacterium, multi-resistant *Staphylococcus aureus*, which is resistant to most antibiotics. Both these types of resistance create the threat of untreatable epidemics developing if not contained.

Identification of the mechanisms underlying the clinical phenomenon of drug resistance to chemotherapeutic agents has predominantly been based on laboratory studies comparing the characteristics of drug sensitive cells with either intrinsic or acquired drug resistant cell lines isolated from primary tumour samples or drug-resistant cell lines derived in the laboratory after chronic exposure to cytotoxic drugs. A spectrum of biochemical and genetic alterations can give rise to cytotoxic drug resistance. Firstly, decreased uptake or increased efflux of cytotoxic drug may result in a decreased concentration of drug at the target site. For example, the over expression of the membrane transport protein, P-glycoprotein (Endicott and Ling, 1989, Gottesman and Pastan, 1993). Secondly, conformationally altered drug targets result in a decrease in drug action. Resistance to methotrexate is associated with alterations in levels of dihydrofolate reductase (DHFR), its target enzyme, either by the production of variant forms of the enzyme (Goldie *et al.*, 1980) or by the overexpression of DHFR by amplified genes (Schimke *et al.*, 1984). The overexpression of topoisomerase I (topo I), the target enzyme for camptothecin (Kjeldsen *et al.*, 1988), or overexpression of topoisomerase II (topo II), the target enzyme for doxorubicin and etoposide (Beck, 1989), is another example of how a decrease in the expression or activity of the drug target protein itself may cause drug resistance. Alteration in the cellular metabolism of cytotoxic drugs by changes in the levels of metabolic enzymes has also been implicated as a mechanism for decreasing drug toxicity. For example, the tripeptide, glutathione (GSH) and its related enzymes are able to react with, and detoxify, many of the reactive alkylating agents used in the clinic (Arrick *et al.*, 1984; Russo *et al.*, 1986). Finally, a novel approach to understanding the mechanisms underlying drug resistance is one involving

investigations into the modulation of cell death by the expression of certain genes. It is possible that some determinants of inherent drug sensitivity and resistance may be independent of those which involve the formation of the drug-target complex and its characteristic biochemical sequelae. A wide variety of antitumour drugs reportedly induce a conserved mode of active death, termed apoptosis, in susceptible cells. Expression of the gene *bcl*-2, which inhibits apoptosis, may make cells resistant to drug-induced apoptosis by a novel mechanism affecting events downstream of the initiation of toxicity brought about by the drug (Fisher *et al.*, 1993).

1.3 Mechanisms of drug resistance

1.3.1 Multidrug resistance (MDR)

As already discussed, to investigate the basis of drug resistance in the laboratory, drug resistant cell lines have been isolated by exposing various drug sensitive cancer cell lines to increasing doses of chemotherapeutic agents. If the selecting agent is one of a group of well defined natural products possessing a number of similar properties, including high molecular weight and lipophilicity, for example doxorubicin, the isolated resistant cells are frequently not only resistant to doxorubicin but may be cross resistant to other, structurally unrelated, natural product drugs with similar properties. This simultaneous resistance to many structurally unrelated drugs has been termed *'classical'* multidrug resistance (MDR) or pleiotropic drug resistance. Over 25 years ago Biedler and Riehm (1970) were first to describe the derivation of cells with a multidrug resistance phenotype. The group of drugs involved in the MDR phenotype includes doxorubicin, vincristine, vinblastine, actinomycin D, colchicine and etoposide but not agents such as cisplatin, cytosine arabinoside, cyclophosphamide and methotrexate, all agents synthesised in the laboratory. The usual pattern of cross resistance includes a large variety of cytotoxic agents that do not have a common structure or a common intracellular target. Although these diverse compounds share no structural similarity, one common feature is that in general, they are all amphiphathic (soluble in both water and lipid) with preferential solubility in

lipid. In addition to the structural dissimilarity, the drugs in the MDR groups have different mechanisms of action; some affect microtubules, some inhibit DNA, RNA or protein synthesis. It is important to remember that MDR is a phenomenon occurring in cells derived in the laboratory. It provides a useful model for the elucidation of the mechanisms involved in drug resistance in the clinic. The following section describes the mechanisms of MDR in detail and examines their individual clinical relevance.

1.3.2 Overexpression of a 170kDa plasma membrane glycoprotein (P-glycoprotein)

(i) Introduction

The first clue to the mechanism(s) underlying MDR came from drug transport studies which demonstrated that MDR cells accumulated less drug than drug sensitive cells (Dano, 1973; Ling *et al.*, 1974). Drug entry into the cell appeared to be normal, but the cells had developed the capacity to pump drugs back into the extracellular matrix. In 1976 Juliano and Ling linked this phenomenon with the expression of a cell surface glycoprotein of 170kDa, known as P-glycoprotein.

Table 1.4 lists classes of agents in clinical or laboratory which are known to interact with P-glycoprotein. This list includes many hydrophobic natural compounds, semi-synthetic analogues of natural products and synthetic organic compounds.

Table 1.4

Classes of agent that interact with P-glycoprotein

ANTICANCER DRUGS	OTHER CYTOTOXIC AGENTS	RESISTANCE MODIFIERS
***Vinca* alkaloids** (e.g.vinblastine) **Anthracyclines** (e.g. doxorubicin) **Epipodophyllotoxins** (e.g. etoposide) **Antibiotics** (e.g. actinomycin D) **Others** (e.g.taxol, mitomycin C) **Modified steroids** (e.g. tamoxifen)	**Antimicrotubule drugs** (e.g. colchicine) **DNA intercalators** (e.g. ethidium bromide) **Toxic peptides** (e.g. valinomycin)	**Calcium channel blockers** (e.g.verapamil) **Antiarrhythmics** (e.g. quinidine) **Immunosuppressants** (e.g.cyclosporine A, FK506) **Steroid hormones** (e.g. progesterone) **Antipsychotics** (e.g. phenothiazines) **Detergents** (e.g. Tween-80) **Antihistamines** (e.g. terfenadine)

(ii) Molecular genetics and nomenclature of P-glycoprotein

Genetic analysis has demonstrated that P-glycoprotein is produced by a small family of homologous genes, known as *mdr* or multidrug resistant genes, which have been cloned and sequenced from human, hamster and murine cells (Endicott and Ling, 1989).

A novel technique, known as in-gel renaturation, which allowed direct cloning of amplified segments of genomic DNA from gels, was used to isolate amplified genomic sequences in MDR hamster cells (Roninson, 1983). This technique was employed after cytogenetic analyses of these highly selected MDR cells indicated the presence of homogeneously staining regions (HSRs) and double minute

chromosomes (Biedler *et al.*, 1970; Fojo *et al.*, 1985). An amplified genomic fragment that detected mRNAs in drug resistant cells was then used to identify and isolate human genomic fragments from multidrug resistant KB cells, which also contained amplified genes (Roninson *et al.*, 1986) and was used as a probe to detect cross-hybridising cDNAs in the mouse (Gros *et al.*, 1986a). More traditional methods based on the overexpression of *mdr* mRNAs and their product, P-glycoprotein have also been used to clone *mdr* cDNAs. The laboratories of Borst and Melera used differential cDNA libraries to isolate cDNAs corresponding to mRNAs overexpressed in highly multidrug resistant Chinese hamster cells (Van der Bliek *et al.*, 1986; Scotto *et al.*, 1986), while Ling's group used a monoclonal antibody to P-glycoprotein (Kartner *et al.*, 1985) to clone a fragment of a cDNA that encoded a hamster P-glycoprotein (Gerlach *et al.*, 1986).

P-glycoprotein is encoded by the *mdr* gene family. Three classes of *mdr* gene have been identified in mammalian cells. Table 1.5 shows the classification of human, mouse and hamster *mdr* genes and their alternative designations (in brackets).

Table 1.5

Classification of mammalian *MDR* isoforms

Pgp Class	Human	Mouse	Hamster	Involved in MDR
I	[a]*MDR 1*	[c]*mdr1a(mdr3)*	[e]*pgp1*	yes
II		[c]*mdr1b(mdr1)*	[e]*pgp2*	yes
III	[b]*MDR3(MDR2)*	[d]*mdr2*	[e]*pgp3*	no

References:

[a]Chen *et al.*, 1986 ; [b]Van der Bliek *et al.*, 1987; [c]Hsu *et al.*, 1989; [d]Gros *et al.*, 1988; [e]Ng *et al.*, 1989

In order to prove that the cloned *mdr* genes were responsible for MDR, several genetic approaches were adopted. Firstly, linkage between *mdr* sequences and drug resistance was demonstrated using gene transfer experiments using high molecular weight DNA (Gros *et al.*, 1986b). After the isolation of full length, functional cDNAs, DNA-mediated transfer (Ueda *et al.*, 1987) or retroviral gene transfer (Guild *et al.*, 1988) showed that the cDNA were able to confer the full phenotype of multidrug resistant cells on drug-sensitive cells. Attempts to show that the human *MDR3* and the closely related mouse *mdr2* can confer multidrug resistance have been negative. Both are present in the liver in the canalicular membranes of hepatocytes. It is thought that they may have a role in phospholipid transport through the canalicular membrane into bile (Smit *et al.*, 1994).

(iii) Structure of P-glycoprotein

The availability of full-length *mdr* cDNAs was quickly followed by their sequencing and the analysis of these sequences. The human MDR genes are adjacent to each other on the long arm of chromosome 7 near 7q21.1 (Reviewed by Trent *et al.*, 1991). P-glycoprotein (c.1280 amino acids in length) is a highly conserved, tandemly-duplicated, molecule joined by a 'linker' sequence of approximately 60 amino acids. Each half of the molecule consists of six transmembrane regions and two putative nucleotide-binding regions. As much as 40kDa of the molecular weight of P-glycoprotein is due to post-translational N-linked glycosylation. Inhibition of this glycosylation does not appear to affect the MDR phenotype (Beck *et al.*, 1982). The sequence of *mdr* cDNAs indicates significant sequence homology with many other energy dependent transport proteins (Gros *et al.*, 1986c). The main regions of sequence homology include the A and B binding folds of the Walker motifs for nucleotide binding and areas immediately between and around these two folds. This suggests that ATPase activity specific to a superfamily of transporters may be encoded in this region. This family is now known as the ATP-binding cassette (ABC) family, of which there are at least 40 members. Examples of proteins within

this family include a pump that mediates chloroquine resistant in the malaria parasite, *Plasmodium falciparum* (Wilson *et al.*, 1989), a transporter for the 'a' peptide mating factor of yeast called STE6 (McGrath *et al.*, 1989) and the cystic fibrosis transport regulator (CFTR), the product of the cystic fibrosis gene (Riordan *et al.*, 1990).

(iv) Post-translational modification of P-glycoprotein

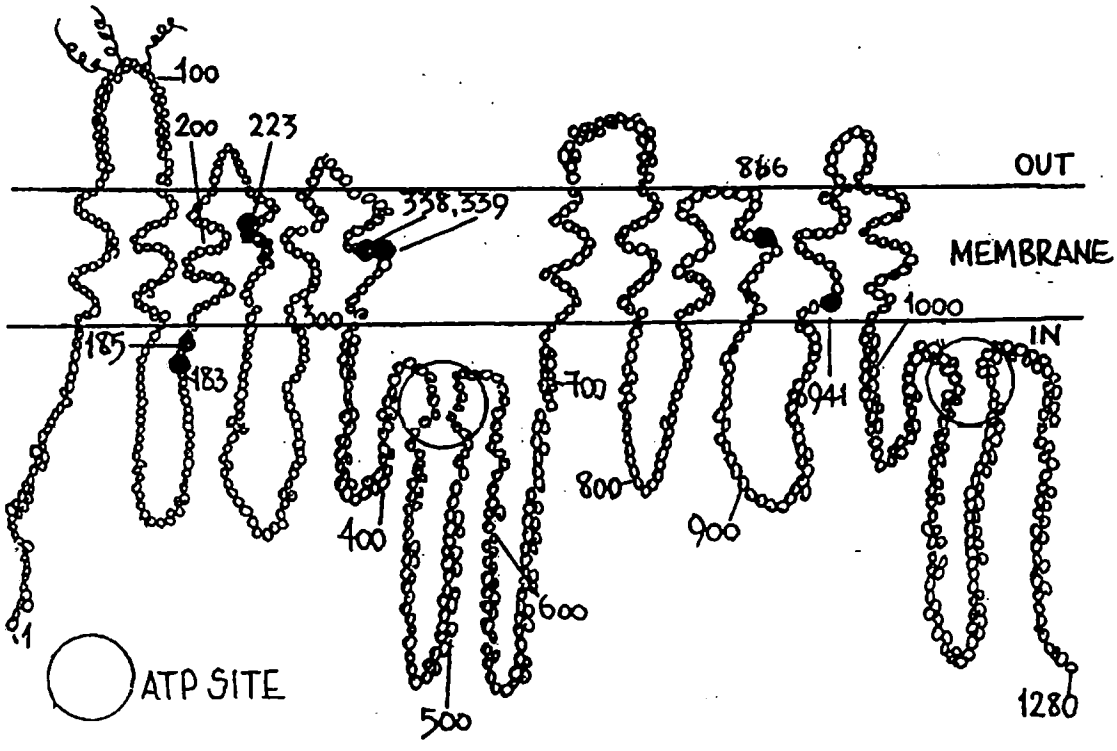
Many groups have been able to show that P-glycoprotein overexpressed in multidrug resistant cells undergoes covalent modification by phosphorylation (Chambers *et al.*, 1990a & b; Bates *et al.*, 1992). Ma *et al.* (1991) demonstrated that P-glycoprotein molecules are subject to rapid cycles of phosphorylation/dephosphorylation. The universal occurrence and dynamic nature of this covalent modification may suggest an important role for phosphorylation in the mechanism of action of P-glycoprotein. This role is discussed at length in Chapter 7 of this thesis.

(v) Analysis of point mutations in P-glycoprotein

The study of mutations occurring in the amino acid sequence of P-glycoprotein has led to speculation about its specific function. There are several examples of point mutations in P-glycoprotein that are known to alter the substrate specificity of the protein. Figure 1.4 is a schematic diagram of the 1280 amino acid structure of P-glycoprotein and indicates the positions of these mutations. The changes in specificity that occur due to these mutations fall into two classes: (a) Mutations that alter the pattern of resistance of the cell, so that resistance to some drugs is increased, and resistance to others is decreased and; (b) mutations that preserve, or slightly increase resistance to a certain class of drug, and decrease resistance to all others. Table 1.6 lists some recently described P-glycoprotein point mutations and reveals the alteration in drug specificity caused by the mutation.

Figure 1.4

A schematic diagram of the structure of P-glycoprotein



The position of several point mutations in the primary structure of P-glycoprotein is superimposed on the secondary structure of the multidrug transporter. Adapted from Gottesman *et al.*, 1993.

Table 1.6

Summary of P-glycoprotein point mutations

SUBSTITUTION AND SITE	ORIGIN	PHENOTYPE
[1,2]Val to Gly @ 185	colchicine selected MDR cell line, KB-C1	increased resistance to colchicine and etoposide, decreased resistance to *vinca* alkaloids, small decrease in resistance to anthracyclines and taxol
[3]Gly to Ala @ 338 Ala to Pro @ 339	Chinese hamster cells, resistant to actinomycin D	increased resistance to actinomycin D, decreased resistance to other drugs
[4,5]Ser to Phe @ 941*	mouse gene mdr1 (mdr 1b)	decreases the transport of colchicine and doxorubicin
[6]Gly to Ala @ 431 or1073* Lys to Arg @ 432 or 1074* (ATP binding sites)	mouse gene mdr1 (mdr 1b)	complete loss of function of the transporter, continued binding of azido-ATP

*represents genetically engineered mutations

References: [1]Choi et al 1991; [2]Kioka *et al.*, 1989; [3]Devine et al., 1992; [4]Hsu *et al.*, 1990; [5]Gros *et al.*, 1991; [6]Azzario *et al.*, 1989

The majority of these mutations occur in or around the transmembrane domains of P-glycoprotein. This may indicate that these regions are important in substrate specificity.

(vi) Identification of expression of P-glycoprotein

The expression of P-glycoprotein protein and mRNA in normal tissues and tumours has been determined in a number of ways. *MDR1* mRNA can be detected by Northern blot or slot blot analysis, by RNAse protection, by *in situ* hybridisation and autoradiography or by a variety of quantitative polymerase chain reaction (PCR)-based assays. Some laboratories have used functional assays based on the ability of P-glycoprotein overexpressing MDR cells to extrude fluorescent dyes, such as rhodamine. If the reduced dye accumulation in MDR cells can then be reversed with a modifier such as verapamil or cyclosporin A, agents known to inhibit the function of

P-glycoprotein, then preliminary, but not definite evidence for the expression of P-glycoprotein can be obtained. Overexpression of P-glycoprotein protein has been measured using antibodies. Table 1.7 shows some of these antibodies and groups them according to their recognition site.

Table 1.7

Antibodies which detect P-glycoprotein

ANTIBODY	RECOGNITION SITE	REFERENCE
C219	internal	Kartner *et al.*, 1985
C494	internal	Kartner *et al.*, 1985
JSB-1	internal	Scheper *et al.*, 1988
MRK-16	external	Hamada *et al.*, 1986
MRK-17	external	Hamada *et al.*, 1986
265/F4	external	Lathan *et al.*, 1985
HYB-612	external	Meyers et al . 1988
MAb 57	external	Cenciarelli *et al.*, 1991
17F9	external	Aihara *et al.*, 1991
4E3.16	external	Arceci *et al.*, 1992
UIC-2	external	Mechetner *et al.*, 1992

The monoclonal antibody C219 is perhaps the most extensively characterised. It binds to its epitope near the ATP-binding site of P-glycoprotein (Georges *et al.*, 1990). C219 is not entirely specific for the *MDR1* product, it also recognises other proteins such as the *MDR2* product and certain forms of myosin. The antibody is, however, useful for immunoprecipitations and Western blotting analysis for semi-quantitative determination of P-glycoprotein levels in tissues (Bell *et al.*, 1985), and for immunohistochemical detection of P-glycoprotein (Chan *et al.*, 1990). In contrast, the monoclonal antibody, MRK-16 has an external recognition site and is quite specific for human P-glycoprotein (Hamada *et al.*, 1986) and is very useful for FACS

analysis or magnetic sorting of P-glycoprotein-bearing cells (Choi *et al.*, 1991). It cannot be used for quantitative immunoprecipitation or Western blots.

(vii) Expression of P-glycoprotein in normal tissues

The first studies on the expression of P-glycoprotein in normal tissues were examinations of *MDR1* gene expression, determined by levels of *MDR1* RNA (Fojo *et al.*, 1987). This was soon followed by immunohistochemical studies using the MRK-16 antibody (Thiebaut *et al.*, 1987). These studies demonstrated there to be very high levels of P-glycoprotein in the adult adrenal gland, the brush border of the renal proximal tubule epithelium, the lumenal surface of biliary hepatocytes, small and large intestinal mucosal cells and pancreatic ductules. Since these first studies, P-glycoprotein expression has been observed in many other tissues, including the brain and testis (Thiebaut *et al.*, 1989), the secretory glands of the pregnant epithelium (Bradley *et al.*, 1990), in placenta, in peripheral lymphocytes (Chaudhary *et al.*, 1992) and in CD34 bone marrow stem cells (Chaudhary *et al.*, 1991). Speculation concerning the normal physiological function of P-glycoprotein has been made based on the known cellular localisation. As P-glycoprotein is expressed in transporting epithelia in kidney, liver, pancreas and intestine and capillary endothelia in the brain and testis its role may involve transepithelial or transendothelial transport of xenobiotics or prevent their absorption. The brain and the testes are known to be pharmacological sanctuaries for metastatic cancer. The presence of P-glycoprotein in the capillaries of these organs may have some significance in terms of protecting these cells from chemotherapeutic damage by transporting cytotoxic drugs out of the cell. P-glycoprotein may serve a blood-tissue barrier function, keeping toxic metabolites and xenobiotics out of these tissues. In the liver P-glycoprotein is found on the biliary surface of hepatocytes, while in the intestinal mucosa and the proximal renal tubule P-glycoprotein is localised to the apical surface of lining epithelial cells, adjacent to the lumen (Thiebaut *et al.*, 1987). The localised distribution of P-glycoprotein in these organs is also suggestive of a membrane transport function. High-level expression in

the adrenal gland suggests a role in steroid secretion or the protection of the membranes of steroid-secreting cells. The observation that progesterone is an inhibitor of P-glycoprotein (Yang *et al.*, 1989) supports this idea.

(viii) Expression of P-glycoprotein in human cancers

Intrinsic expression of the *MDR1* gene is found in cancers derived from tissues that normally express the *MDR1* gene such as, kidney, liver, colon, pancreas and adrenal gland (Fojo *et al.*, 1987). Other untreated cancers such as acute and chronic leukaemia of children and adults, non-Hodgkin's lymphoma, chronic myelogenous leukaemia in blast crisis, non small cell lung cancer with neuro-endocrine properties, neuroblastoma, sarcoma and astrocytoma express high levels of *MDR1* RNA but originate from tissues that do not express *MDR1* RNA at easily detectable levels (Gottesman *et al.*, 1993). This observation has led to the suggestion that the process of malignant transformation may activate expression of the *MDR1* gene. This is supported by two studies. One study demonstrated that *ras* and mutant p53, two genes commonly associated with tumour progression, were able to stimulate the *MDR1* promoter (Chin *et al.*, 1992). Another study reported increased *MDR1* gene expression at invading margins of locally aggressive and metastatic cancers (Weinstein *et al.*, 1991). *MDR1* gene expression is often observed in tumours that have relapsed during the course of, or after, chemotherapy. Examples of these include lymphoma, leukaemia, neuroblastoma, cancers of the breast and ovary, and multiple myeloma (Dalton *et al.*, 1989). This may indicate that either there was a small pre-cursor population of *MDR1* expressing cells that survived the initial treatment and then regrew to produce a drug resistant relapsed tumour or that the chemotherapy itself induced *MDR1* gene expression.

(ix) Mechanisms of P-glycoprotein-mediated multidrug resistance

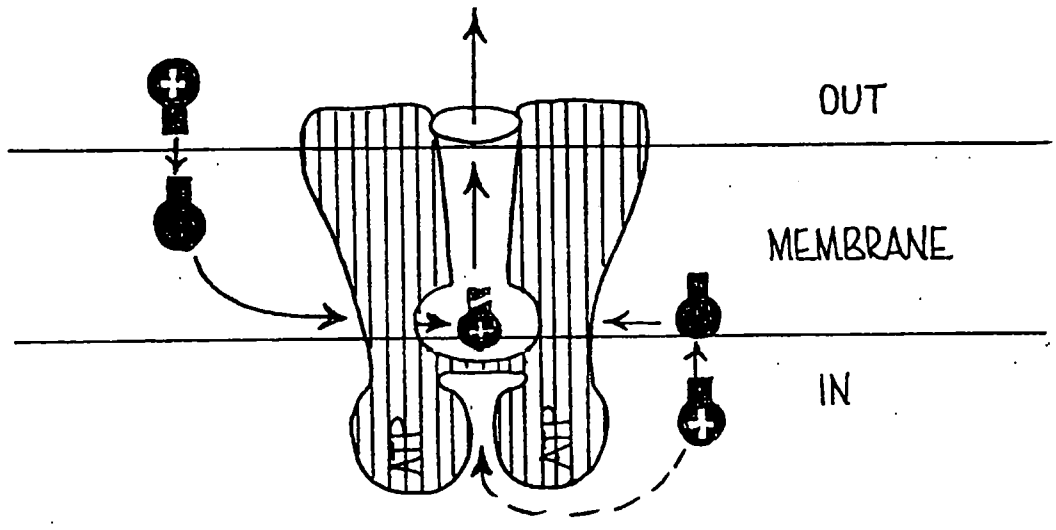
A interesting question with respect to MDR is how a single transporter can transport such a broad range of structurally heterogeneous substrates. It is difficult to imagine

an enzyme-like binding site with the affinity for diverse substrates such as anthracyclines, alkaloids, actinomycin D, cyclic and linear peptides as well as an assortment of other hydrophobic and amphiphathic compounds.

Before the demonstration of the ATP-dependent efflux pump activity of P-glycoprotein (Willingham *et al.*, 1986), drug resistance was attributed to a reduction in the permeability of the plasma membrane to certain drugs (Ling *et al.*, 1974). When the efflux pump activity of P-glycoprotein was identified, this was thought to be the sole mechanism by which decreased intracellular drug accumulation was achieved. It has more recently been demonstrated that the multidrug resistance transporter is able to reduce influx of drug into the cytosol as well as increase efflux. Taking this fact into consideration the most popular current model for the mechanism of action of P-glycoprotein is one based on the removal of drug directly from the plasma membrane. This model, adapted from Gottesman *et al.*, 1993, is shown in figure 1.5. This type of model has been named the "hydrophobic vacuum cleaner" and has two major features:

Figure 1.5

P-glycoprotein-"The hydrophobic vacuum cleaner" model



A possible mechanism of action for P-glycoprotein emphasising 2 features: (1) drugs may be detected in and ejected from the plasma membrane; and (2) the transporter has a 3D structure involving one or more p170 subunits, which bring both halves together to form a single transporter channel. Adapted from Gottesman *et al.*, 1993.

Firstly, drugs can be detected and expelled as they enter the plasma membrane.

The series of structurally diverse compounds belonging to the MDR phenotype are amphiphathic compounds that are preferentially soluble in lipid (hydrophobic). In this model, the primary determinant of specificity would be the ability of the substrate to intercalate into the lipid bilayer (i.e. hydrophobicity). Interactions with the substrate binding site would be of secondary importance, although they would still play a role in determining specificity. Hydrophilic drugs would not intercalate in the lipid bilayer in an appropriate fashion and would therefore not be substrates for the MDR transporter. This may provide an explanation for the unusual transport kinetics associated with P-glycoprotein-mediated transport. If the substrate is extracted from the lipid bilayer, the actual concentration of drug that comes into direct contact with the protein is unknown and will be different from the concentration of the drug added to the cellular matrix (Higgins *et al.*, 1992a & b). There are two sets of data in support of the idea that drugs are transported directly from the plasma membrane. Firstly, Kessel *et al.*, demonstrated that rhodamine 123, a highly fluorescent mitochondrial laser dye which is an excellent P-glycoprotein substrate (Neyfakh *et al.*, 1988), has a different fluorescence excitation spectra in drug sensitive and resistant cells. In sensitive cells the excitation spectrum of rhodamine 123 resembles its spectrum in octanol, suggesting that the dye lies within a highly hydrophobic environment such as the plasma membrane. In contrast in resistant cells, the spectrum looks more like that of rhodamine in aqueous environment, suggesting that the drug has been removed from the plasma membrane (Kessel *et al.*, 1989). Localisation of rhodamine 123 in sensitive and MDR cells using confocal microscopy has confirmed this result (Weaver *et al.*, 1991). Secondly, Raviv *et al.*, in 1990 showed that by measuring the transfer of energy from doxorubicin to iodinated naphthalene azide, a highly hydrophobic label of membrane constituents including transmembrane proteins, the presence of doxorubicin within the membranes of drug sensitive cells could be demonstrated. In drug resistant cells, however, doxorubicin

was only found in the plasma membrane associated with P-glycoprotein. This result indicates that the MDR transporter has removed doxorubicin from the plasma membrane (Raviv *et al.*, 1990)

Sencondly, transport occurs through a single channel of the transporter.

The main evidence for the second feature of this model for the mechanism of the MDR transporter, a single transport channel, comes from binding and photoaffinity labelling data (Bruggeman *et al.*, 1992). Bruggeman was able to demonstrate that inhibition of azidopine labelling by vinblastine reduces labelling equivalently in both the amino- and the carboxy-terminal halves of P-glycoprotein, suggesting that these two binding sites are equivalent with respect to their ability to bind two different drugs. These data support a model for P-glycoprotein in which both halves of the transporter come together to form a single transport channel. Although it has also been shown recently that the precise binding sites of verapamil, a modifier of MDR, and vinblastine are not identical this does not necessarily provide negative evidence for the theory of single channel transport. The transport channel for drugs, identified by the photoaffinity labels of Bruggeman, and the initial drug binding sites, which probably define how drugs enter the transporter may well be different (Gottesman *et al.*, 1993).

It is well documented that the process of drug transport by P-glycoprotein is active and ATP-dependent (Horio *et al.*, 1988). We know very little, however, concerning the way in which the energy of ATP is harnessed by the transporter. It has been demonstrated that both ATP sites are required for ATP-dependent drug transport to occur efficiently (Azzaria *et al.*, 1989) and that drugs are themselves capable of stimulating ATPase activity (Sarkardi *et al.*, 1992; Ambudkar *et al.*, 1992). In order to explain how the energy of ATP is transduced to result in the transport of drugs from the plasma membrane to the extra cellular matrix, Higgins *et al.*, introduced the

idea that P-glycoprotein acts as a "flippase". In this model the drug is flipped from the inner leaflet of the lipid bilayer to either the outer leaflet of the lipid bilayer or the extra cellular medium (Higgins *et al.*, 1992b). Little is known about mechanisms of phospholipid flippases, therefore further experimentation is required before this idea can be supported or refuted.

An alternative model for the mechanism of MDR proposes that the characteristic reduced intracellular accumulation of drug in MDR cells is a result of perturbations in a variety of complex drug/cell interactions that are essentially precipitated by alterations in the character and magnitude of the plasma membrane electrode potential. Consistent with this model are many intracellular pH and plasma membrane electrical potential perturbations in MDR cells (Thiebaut *et al.*, 1990; Roepe *et al.*, 1993; Luz *et al.*, 1994; Wei *et al.*, 1994). Irregularities in chloride conductance, also in turn influence electrochemical potential (Gill *et al.*, 1992). This model essentially suggests that P-glycoprotein may act as an intracellular pH regulator, possibly acting to sequester drug molecules into specific subcellular compartments to mediate efflux or to affect pH-dependent binding of drugs to cellular targets (Roepe *et al.*, 1992 & 1993). An elegant study carried out by Ruetz and Gros (1994) contradicts the evidence in support of this type of model. In this study the group examined drug uptake into inside-out plasma membrane or secretory vesicles made from *S. cerevisiae* harbouring overexpressed murine mdr3 protein. They found that, in *mdr3*-expressing secretory vesicles, vinblastine accumulation was not affected by the presence or absence of the membrane potential. In addition, they demonstrated that *mdr3* caused an enhanced accumulation of the lipophilic cation and P-glycoprotein substrate tetraphenylphosphonium in secretory vesicles, despite the presence of a strong transmembrane proton gradient. The group conclude that P-glycoprotein mediated drug transport is not only independent of a proton gradient but is also not coupled to proton movement across the membrane of the secretory vesicle (Ruetz *et al.*, 1994a&b).

A further speculative model was proposed by Gill and co-workers in 1992. This model suggests that P-glycoprotein is bi-functional, serving not only as a active drug transporter but also as a chloride channel activated by cell swelling. This suggestion was based on a number of observations. Firstly, hypotonicity-induced chloride conductances correlated with P-glycoprotein expression. Secondly, inhibitors of drug transport, including verapamil, inhibited hypotonicity-induced chloride conductances. Finally, site-directed mutations in the nucleotide-binding folds of P-glycoprotein, which prevent ATP hydrolysis but preserve ATP binding, eliminate drug transport dependent on ATP hydrolysis but did not effect chloride channel function dependent on ATP binding. Cystic fibrosis transmembrane regulator (CFTR), the protein product of the cystic fibrosis gene and another member of the ABC superfamily of transporters, is a cyclic AMP-dependent chloride channel. The fact that bifunctionality could, therefore, be a characteristic of CFTR and other ABC transporters makes this model even more intriguing. In contrast the findings of another group, Dong *et al.*, (1994), completely contradict those of Gill *et al.* They conclude that despite large differences in P-glycoprotein expression levels they found no differences in hypotonicity-induced chloride currents among three drug sensitive cell lines and their MDR sublines. They were also unable to demonstrate evidence of chloride current blockade by the resistance modifier, verapamil. Three other groups also provided contradictory evidence. Wang *et al.* (1994) found no difference in hypotonicity-induced chloride currents between the drug sensitive T-lymphoblastic cell line, CCRF-CEM and its classical MDR subline CEM/VLB100. Altenberg *et al.* (1994a&b) and Weaver *et al.* (1993) demonstrated that P-glycoprotein mediated active transport and electrodiffusive chloride transport are dissociated.

(x) Reversal of P-glycoprotein mediated multidrug resistance

A number of approaches have been used to circumvent the problem of multidrug resistance in laboratory models. One such approach has been the use of additional

chemical compounds, resistance modifiers which, when co-administered with a cytotoxic drug lead to a partial or complete restoration of sensitivity in resistant cells. Tsuruo *et al.*, in 1981 provided the first evidence of a compound capable of restoring sensitivity to resistant cells. The group demonstrated that verapamil, a calcium channel blocker, was able to sensitise vincristine-resistant cells to vincristine and vinblastine. This was observed *in vitro* and in mice bearing Ehrlich ascites tumours resistant to vincristine. During the early 1980s verapamil was studied with great interest. It was originally thought that the increase in efflux of cytotoxic drugs observed in MDR cells may be linked to calcium transport and so calcium channel blockers should therefore restore sensitivity to these cells. Verapamil has, however, been shown to be an active modifier of MDR even in MDR cells which lack calcium channels. It has now been shown that verapamil is able to bind directly to P-glycoprotein (Safa *et al.*, 1988) and its mode of resistance modification is thought to be via competitive inhibition of P-glycoprotein (Zamora *et al.*, 1988; Tsuruo *et al.*, 1981). The calcium channel blocking activity of verapamil is now considered to be unrelated to its resistance modifying action. It has been shown, however, to enhance ATP consumption (Broxterman *et al.*, 1988), to restore cytoplasmic pH (Vayuvegula *et al.*, 1988), to correct decreased membrane potential and to increase intranuclear concentrations of doxorubicin and daunorubicin in MDR cells (Schuurhuis *et al.*, 1989). This may indicate that the mechanisms underlying the way in which verapamil reverses MDR are rather more complexed than simple competitive inhibition of P-glycoprotein.

During the 1980s, after the discovery of verapamil as a resistance modifier, other compounds including other calcium channel blockers and calmodulin inhibitors were demonstrated to have resistance modifying properties. Analysis of common physical-chemical properties shared by compounds that modulate MDR revealed that many of these agents had two planar aromatic domains and a basic nitrogen (Pearce *et al.*, 1990). Structural similarities may play a role in the ability of some compounds to

modulate multidrug resistance but it appears that lipid solubility, physiological pH, cationic charge and molar refractivity are more important physiological properties of modulators (Zamora *et al.*, 1988). The immunosuppressive agent, cyclosporin A, was initially identified as an effective resistance modifier due to its interaction with calmodulin. Slater *et al.* (1986a & b) were the first group to show the activity of cyclosporin A as a resistance modifiers. Cyclosporin A has a particularly high binding affinity to P-glycoprotein. Like verapamil, it may also inhibit P-glycoprotein competitively. Twentyman *et al.* (1987a & 1988) examined the relationship between immunosuppression and resistance modification for a number of cyclosporin A analogues. They found that chemically-modified cyclosporins could be non-immunosuppressive whilst being highly effective resistance modifiers (Twentyman *et al.*, 1988). There are now several examples of 'second generation' chemosensitisers with high molar potency for reversing MDR. Studies carried out at Sandoz led to the identification of one such compound, PSC-833, a novel cyclosporin analogue, which is a lead compound for clinical trial as a resistance modifier (Gaveriaux *et al.*, 1991). A novel approach to circumvention of MDR has been to combine modifiers. The rationale behind this strategy is to increase the therapeutic index of chemosensitisation by combining agents that produce a positive interaction in reversing MDR but differ in dose limiting toxicities. *In vitro*, various combinations of resistance modifiers have demonstrated a synergistic interaction. For example, verapamil in combination with either quinidine (Lehnert *et al.*, 1991) or cyclosporin A (Hu *et al.*, 1990). In addition to the use of drugs to circumvent the phenomenon of MDR other agents such as antibodies (Hamada *et al.*, 1986; Merchetner *et al.*, 1992), immunotoxins (Fitzgerald *et al.*, 1987), antisense oligonucleotides (Thierry *et al.*, 1993) and liposome encapsulated drugs (Mickisch *et al.*, 1992) have all been shown to be valid approaches to the elimination of MDR *in vitro*. Further investigations and *in vivo* studies are required before their clinical potential can be evaluated. Modifiers of P-glycoprotein mediated MDR are characterised and discussed in more detail in Chapter 4.

(xi) Clinical trials

On the basis of experimental evidence of the importance of the concept of MDR reversal in overcoming chemotherapeutic refractiveness, several agents with the capability to reverse multidrug resistance have entered clinical trial over the last few years. For example verapamil (IV and oral), D-verapamil, cyclosporin A, quinidine sulphate, trifluoperazine and tamoxifen. Table 1.8 summarises the outcome of some of the earlier trials.

Table 1.8

Examples of the outcome of early clinical trials involving resistance modifiers

THERAPY	MALIGNANCY & PATIENT NO.	REMISSIONS COMPLETE (PARTIAL)	REFERENCE
Verapamil (Doxorubicin)	Ovarian (8)	0(0)	Ozols *et al.*, 1987
Verapamil (VAD*)	Myeloma (22)	0(5)	Salmon *et al.*, 1991
Verapamil (VAD+cyclophos.)	non-Hodgkins lymphoma(14)	4(7)	Miller *et al.*, 1991
D-verapamil (Doxorubicin)	Colorectal (15)	0(1)	Chabner *et al.*, 1990
Trifluoperazine (Doxorubicin)	Various (36)	1(6)	Miller *et al.*, 1988
Cyclosporin A (Vinblastine)	Renal (15)	0(0)	Rodenburg *et al.*, 1991

*VAD-vincristine, doxorubicin, dexamethasone

Verapamil was the first and most extensively studied of the MDR modulators. Cardiotoxicity proved to be the dose-limiting toxicity at concentrations of 1-2μM, whereas in most MDR models, concentrations of 6-10μM are required to inhibit fully P-glycoprotein. The D- and L- isomers of verapamil are equally effective as P-glycoprotein inhibitors (Damiani *et al.*, 1993). The D-isomer, however, has considerably less cardiovascular effects. The use of D-verapamil has permitted

modest increases in achievable serum levels (about 2μM), although cardiovascular effects remain dose-limiting. The main metabolite, norverapamil, is also an effective modulator of MDR. The combined serum effects of D-verapamil and its norverapamil metabolite may reach levels sufficient to reverse MDR. Tolcher *et al.* (1994) found that D-verapamil administration resulted in a 2-fold increase in the area under the curve (AUC) of paclitaxel (i.e. cytotoxicity of paclitaxel) without any effect on paclitaxel-induced toxicity. Dexniguldipine, a new calcium channel blocker analogue, is currently being evaluated in phase I trials. *In vitro*, dexniguldipine effectively reverses MDR at concentrations of 1-2μM. Steady state serum levels of 0.5μM have been achieved, with bradycardia as the dose limiting toxicity (Scheulen *et al.*, 1994). To date, no significant alterations in the pharmacokinetics of cytotoxic drugs involved in the MDR phenotype have been reported with dexniguldipine *in vivo*.

There have been numerous clinical trials using cyclosporin A as a modulator of MDR. These trials were preceded by two case reports showing evidence for the activity of cyclosporin A in combination with cytotoxic drugs *in vivo*. Firstly, in 1985, Kloke and Osieka documented a case in which a patient with etoposide-refractory T-cell acute lymphocytic leukaemia (ALL) was given cyclosporin A in combination with etoposide, with the aim of selectively suppressing T-cell growth with the cyclosporin A. The combination resulted in a rapid decrease in circulating leukaemic cells and prolonged myelosuppression (Kloke *et al.*, 1985). Secondly, Sonneveld described the use of cyclosporin A and daunorubicin in the treatment of a patient with acute myelocytic leukaemia (AML). Circulating AML cells were found to be MDR1 positive. Treatment of the patient with daunorubicin and cyclosporin A resulted in the elimination of the MDR1 positive cells (Sonneveld *et al.*, 1992). Unlike trials with verapamil, most of the cyclosporin A studies have documented marked effect on the pharmacokinetics of cytotoxic drugs and significant enhancement in chemotherapy-related toxicities (Linn *et al.*, 1994; Bartlett *et al.*, 1994; Fisher *et al.*, 1994a). The

cyclosporin analogue, PSC 833, has emerged as a non-nephrotoxic, non immunosuppressive agent that is 5- to 10-fold more potent than cyclosporin A as a modifier of MDR. Phase I studies have indicated that serum concentrations of 0.5 to 1μM can be achieved with intravenous (Boote *et al.*, 1994) and oral (Fisher *et al.*, 1994b) formulations. Dose-limiting toxicity is neurologic, with reversible ataxia predominating (Fisher *et al.* 1994b). Marked pharmacokinetic effects have been observed even at lower doses of PSC 833. Like cyclosporin A, increased myelosuppression requires an approximate 2-fold reduction in the administered dose of cytotoxic drug when given in combination with PSC 833. The prolonged half-life of MDR-related cytotoxic drugs when administered in combination with PSC 833 or cyclosporin A is likely to be due to impaired elimination via a direct inhibition of P-glycoprotein in the liver and to a lesser extent in the kidney.

It is clear that the best responses in the early verapamil and cyclosporin A trials (Table 1.8) were achieved in the treatment of malignant lymphomas, leukaemias and multiple myeloma (Raderer *et al.*, 1993). There are several reasons why these initial clinical trials have indicated a predominantly negative outcome. Firstly, many of the modulators used in these clinical trials could not be administered at an optimal concentration due to inherent toxicity. For example, verapamil causes cardiac toxicity. Secondly, many of the clinical trials have been performed in an uncontrolled fashion involving only a small number of patients, sometimes with differing tumour types. These patients often have very advanced tumours making the chance of a response even smaller. In addition, most trials with agents that modify MDR have included patients with unknown P-glycoprotein expression and several phase II studies have been performed in tumours in which it is unlikely that the *MDR1* gene product is a major cause of drug resistance, such as, small cell lung cancer or ovarian cancer (Raderer *et al.*, 1993). Establishing the P-glycoprotein status of the tumour is especially important in order to correlate modifier activity with a direct effect on P-glycoprotein function. Another reason for the disappointing therapeutic outcome may

be that the expression of P-glycoprotein may be heterogeneous within a given population of tumour cells (Rothenberg *et al.*, 1989) or mutant forms of P-glycoprotein may exist that may not be affected by agents that modulate the *MDR1* gene product (Herzog *et al.*, 1992). Finally, other mechanisms of resistance may co-exist with the occurrence of P-glycoprotein in MDR cells. They may also exist in cells lacking P-glycoprotein causing resistance independently. These include changes in the levels of activity of the important nuclear target enzyme topoisomerase II (Beck, 1989) and increased levels of intracellular GSH and glutathione-*S*-transferase, both of which act to increase detoxification of agents such as anthracyclines (Cowan *et al.*, 1986).

Phase I trails of currently available MDR modulators have yielded important pharmacological principles relating to the normal function of anticancer drugs involved in the MDR phenotype. Although these modulators lack the potency to reverse completely MDR at clinically achievable concentrations, encouraging clinical results have been obtained in the haematological malignancies.

1.3.3 Overexpression of the multidrug resistance associated protein (MRP)

(i) Introduction

Resistant tumour cells have been described that appear to express a 'classical' MDR phenotype (decreased cellular accumulation of drugs, altered cellular drug distribution, broad cross-resistance pattern) but do not overexpress the MDR1 gene product P-glycoprotein.

(ii) The discovery of MRP

In 1988, Melvin Center was the first person to show that a surface membrane protein distinct from P-glycoprotein contributed to reduced cellular accumulation in

HL60/ADR leukaemia cells (McGrath *et al.*, 1988). It was not until 1992 that this membrane protein was shown to be identical to the multidrug resistance associated protein (MRP) gene product. The MRP gene which encodes for a 190kDa protein, was first cloned in the multidrug resistant H69/AR, small cell lung cancer cell line by Susan Cole and co-workers (Cole *et al.*, 1992a). The MRP protein has recently been the subject of intense research interest due to the discovery that MRP is identical to the GSH conjugate transporter present in hepatocytes, macrophages and other normal cell types (Jedlitschky *et al.*, 1994).

Isolation and sequencing of the MRP gene was carried out using cloned cDNAs which corresponded to mRNA highly expressed in the small cell lung cancer (SCLC) MDR cell line, H69/AR (Cole *et al.*, 1992a). The primary sequence and predicted size (1531 amino acids) of the MRP gene, located on chromosome 16 (16p13.1), indicated that it is a member of the ATP-binding cassette (ABC) transporter superfamily (Hughes *et al.*, 1994). MRP possesses several hallmarks of ABC proteins - it is a bipartite molecule comprising 8 hydrophobic transmembrane regions in its N-terminal half and 4 in its C-terminal half, with both portions of the molecule containing nucleotide binding folds at their C-terminal ends (Figure 1.6). Despite overall structural similarities, comparison with other family members shows only distant amino acid identity with known ABC proteins (P-glycoprotein, 14% & CFTR, 17%; Grant *et al.*, 1994), identifying most closely with the leishmania P-glycoprotein-related molecule with most identity lying in the nucleotide binding regions. These findings make MRP an obvious candidate for the putative drug transporter in non-P-glycoprotein multidrug resistance cells. In order to confirm that MRP is directly responsible for the multidrug resistance (decreased drug accumulation) observed in these non-P-glycoprotein cell lines, transfection studies were carried out. The stable transfection of both Hela cells (Grant *et al.*, 1994) and the non-small cell lung cancer (NSCLC) SW-1573 cells (Zaman *et al.*, 1994) demonstrated that MRP increases the resistance to natural product cytotoxic drugs

such as doxorubicin, vincristine, vinblastine, etoposide and to a lesser extent taxol (Cole *et al.*, 1994). However, the issue is confused by the fact that the H69/AR cell line, is one of the few cell lines without an accumulation deficit (Cole *et al.*, 1992a & b). This indicates that at least in this system MRP does not contribute to MDR as a membrane drug efflux pump similar to P-glycoprotein. In addition, some MRP expressing MDR cell lines also demonstrate altered topoisomerase II activity . These data indicate that multiple resistance mechanisms may operate in MDR cells that express MRP or other transport proteins. Table 1.9 summarises data on a number of the well characterised MRP-overexpressing cell lines.

Table 1.9

MRP overexpressing human MDR tumour cells lines

CELL LINE (TYPE)	SELECTING DRUG	DECREASED DRUG ACCUMUL	REFERENCE
COR-L23/R (NSCLC)	doxorubicin	yes	Barrand *et al.*, 1994
MOR/0.4R (adenocar. lung)	doxorubicin	yes	Barrand *et al.*, 1994
SW-1573/1R120 (NSCLC)*	doxorubicin	yes	Baas *et al.*, 1990
MCF7/VP (breast cancer)	VP-16	yes	Schneider *et al.*, 1995a
GLC4/ADR (SCLC)*	doxorubicin	yes	Meijer *et al.*, 1991
H69/AR (SCLC)*	doxorubicin	no	Cole *et al.*, 1992
HL60/ADR (leukaemia)	doxorubicin	yes	Krishnamachary *et al.*, 1993
HL60/AR (leukaemia)	doxorubicin	yes	Lutzky *et al.*, 1989
U-937-A10 (leukaemia)	doxorubicin	yes	Slapak *et al.*, 1994

*(N)SCLC-(Non) Small Cell Lung Cancer

Figure 1.6

The structure of MRP

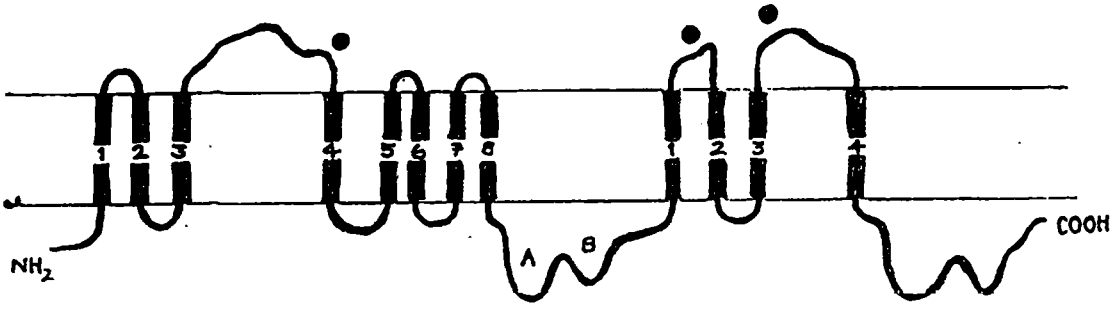


Figure 1.6 demonstrates the predicted structure of MRP. Analysis of the deduced amino acid sequence of MRP predicts that the NH_2-proximal half of the molecule may contain 8 or more transmembrane segments followed by a nucleotide binding domain (NBD1).

The COOH-proximal half of the molecule may contain only 4 transmembrane segments followed by a second nucleotide binding domain (NBD2).

A and B, motifs are characteristic of nucleotide-binding domains of ABC transport proteins.

•, potential N-glycosylation sites that are predicted to be on the outside of the membrane.

(Adapted from Almquist *et al.*, 1995)

(iii) Expression of MRP in normal human tissues

Using RNase protection analysis, expression of MRP mRNA was found to be widespread in all normal tissues analysed, including kidney, stomach, lung, intestine, thyroid, bladder, testis, adrenal gland, pancreas, placenta, liver, ovary and muscle (Zaman *et al.*, 1993). Localisation of MRP on a cellular level was examined by Thomas *et al.* (1994). The group localised MRP on histological tissue sections using *in situ* hybridisation and demonstrated the high expression of MRP in normal bronchial epithelial cells. Initial data obtained using recently available monoclonal antibodies (Flens *et al.*, 1994; Hipfner *et al.*, 1994) confirms the results of Thomas *et al.* MRP expression was also observed in the adrenal cortex and the epithelial cells of the GI tract.

(iv) Mechanism(s) of MRP-mediated MDR

Like P-glycoprotein, MRP is capable of actively transporting cytotoxic drugs out of cells against a concentration gradient (Versantvoort *et al.*, 1992). Furthermore, transfection of MRP into drug sensitive tumours cells demonstrated that MRP caused the efflux of drugs such as daunorubicin against a concentration gradient in transfected cells (Zaman *et al.*, 1994). Conversely the H69/AR cell line in which MRP was originally cloned (Cole *et al.*, 1992a & b) does not show a decreased drug accumulation. Susan Cole explains this phenomenon by hypothesising that MRP, apart from being a plasma membrane transporter, may be capable of pumping drugs into cytoplasmic vesicles in preparation for removal by exocytosis (Cole *et al.*, 1992a & b). MRP is localised on the plasma membrane of tumour cells (including the MRP-overexpressing, laboratory derived, multidrug resistant cells). In highly MRP overexpressing cells a golgi-like localisation is also observed. This difference in MRP localisation may be important in terms of MRP mechanism. The observation of MRP expression on a golgi-like structure may explain why no intracellular accumulation deficit is observed in Susan Cole's H69/AR cells. Versantvoort *et al.* (1994) demonstrated that daunorubicin transport in MRP overexpressing cells was

saturable, dependent on ATP concentration in the millimolar range and could be competitively inhibited by the tyrosine kinase inhibitor, genistein. It is fairly clear that MRP induces ATP-dependent removal of drug from tumour cells. As in the case of P-glycoprotein, the mechanism for this active transport has not been precisely defined.

It has recently been demonstrated that MRP is identical to the GSH conjugate transporter present in hepatocytes, macrophages and other normal cell types (Jedlitschky *et al.*, 1994). Thomas *et al.* (1994) demonstrated high level expression of MRP mRNA in normal human bronchial epithelium and at the advancing edge of a variety of lung tumour types. Interestingly the leukotriene LTC_4, the GSH conjugate of leukotriene LTA_4, is transported in an ATP-dependent manner into membrane vesicles prepared from the MRP-overexpressing multidrug resistant cell line HL60/ADR (Leier *et al.*, 1994a & b). The LTC_4 transport into these vesicles was competitively inhibited by the leukotriene LTD_4 receptor antagonist MK571, an anionic quinoline derivative (Jedlitschky *et al.*, 1994; Leier *et al.*, 1994a&b). Photo affinity labelling using [^{3}H]LTC_4 identified a distinct overexpression of a 190kDa protein species in membranes prepared from the HL60/ADR cells. This labelling was efficiently competed by MK571 (Jedlitschky *et al.*, 1994; Leier *et al.*, 1994a&b). These results together with the observed overexpression of MRP mRNA in human bronchial epithelium may indicate a normal physiological role for MRP as a transporter of GSH conjugates, specifically GSH conjugated leukotrienes in the lung. The relationship between GSH biochemistry and the activity of MRP has been further investigated by Versantvoort *et al.*(1995a) Depletion of cellular glutathione by DL-buthionine S,R-sulphoxamine (BSO) in cells which overexpress MRP, resulted in a decrease of daunorubicin and rhodamine 123 efflux. Restoration of cellular GSH levels by the addition of GSH ester to the medium restored the efflux activity. The mechanism of action of MRP and its link with cellular GSH metabolism is currently the subject of intense research interest and is discussed further in Chapter 6.

(iv) Clinical relevance of MRP

It is difficult to speculate about the clinical relevance of MRP due to the lack of detailed data on the expression of MRP in human primary tumour samples. Evidence is emerging that MRP mRNA may be expressed in acute myeloid leukaemia (AML) and multiple myeloma, although only at the baseline levels observed in normal lymphocytes. The results showed as much as a 10-fold variation between individual AMLs. These studies have also demonstrated that their is no relationship between the expression of P-glycoprotein and MRP (Burger *et al.*, 1994; Hart *et al.*, 1994; Schneider *et al.*, 1995). This compounds the problem of reversal of MDR as both P-glycoprotein and MRP levels would need to be determined before treatment could be considered. Agents that inhibit MRP-mediated drug transport are still in the developmental stage. Possible ways of modifying MRP -mediated MDR are discussed in detail in Chapters 5 and 6. More recently, MRP overexpression has been observed in chronic lymphocytic leukaemia (CLL) (Burger *et al.*, 1995; Hart *et al.*, 1995) and prolymphocytic leukaemia (PLL) ((Burger *et al.*, 1995).

(v) Other membrane transport proteins associated with MDR

There are several reports of other non-P-glycoprotein cell lines that express drug transporter proteins other than P-glycoprotein or MRP. The majority of these novel transporters have not yet been identified nor have the cell lines been fully characterised. One protein that has been identified, is the 110kDa product of the LRP gene. Overexpression of this 110kDa protein has been measured in many drug resistant cell lines and is often co-expressed with the MRP protein (Schepler *et al.*, 1993). The protein is recognised by the monoclonal antibody LRP56, which was raised against the non-P-glycoprotein MDR cell line, SW-1573/1R50. The drug sensitive cells showed the same staining pattern with LRP56, localised in a punctate cytoplasmic fashion, but the intensity of the staining was less. LRP protein (originally named Lung Resistance-related Protein) overexpression has been found to

predict a poor response to chemotherapy in acute myeloid leukaemia (List *et al.*, 1993) and ovarian carcinoma (Izequierdo *et al.*, 1994). Scheffer *et al.* (1995) cloned the *LRP* gene and localised it to chromosome 16, close to the *MRP* gene. The deduced amino acid sequence shows a 87.7% identity with the 104kDa rat major vault protein. Vaults are multi-subunit structures that may be involved in nucleo-cytoplasmic transport (Scheffer *et al.*, 1995). The frequent up regulation of the *LRP* gene in MDR tumour cells together with its value in predicting *in vitro* and clinical response to chemotherapy support the view that LRP and/or vaults can, when co-expressed with members of the transmembrane transporter family, contribute to cytostatic drug resistance.

1.3.4 Drug resistance mediated by drug metabolising and detoxifying enzymes

The tripeptide, glutathione (GSH) is the principal cellular non-protein thiol and is able to react with, and detoxify, many of the reactive alkylating agents used in chemotherapy (Arrick *et al.*, 1984; Russo *et al.*, 1987). This interaction is catalysed by a family of enzymes known as glutathione-*S*-transferases (GST). In addition, the enzyme involved in the oxidation of GSH to the disulphide GSSG, glutathione peroxidase (GPX), can detoxify free radicals including those produced in DNA by a number of cytotoxic drugs (including alkylating agents and doxorubicin) and also reduce toxic peroxides (Arrick *et al.*, 1984; Russo *et al.*, 1987).

Modification of GSH metabolism has been proposed as a mechanism leading to resistance to a range of anticancer drugs, and under some conditions, radiation (Moscow *et al.*, 1988). Stepwise selection of sublines of cells for increasing resistance to cisplatin or to alkylating agents such as melphalan has resulted in cells with increased levels of GSH. For example, the melphalan resistant tumour cell line L1210, shows increased GSH compared to the parent line (Suzukaka *et al.*, 1986). It is important to note that this type of resistance cannot be referred to as 'classical

MDR' since the cell lines are only cross resistant to a limited group of compounds such as alkylating agents and platinum analogues. Resistance in these sublines can be reversed as a result of inhibition of GSH by buthionine sulphoximine (BSO), an inhibitor of GSH synthesis or by depletion of cellular levels of GSH by agents which directly inactivate it, such as diethylmaleate (Russo *et al.*, 1987). Some cell lines selected for resistance to alkylating agents or to doxorubicin have increased activity of one or more GST isoenzymes (Kramer *et al.*, 1988). Many researchers believe that in order for a gene product to be causatively linked to drug resistance, transfection studies must be performed. This area has provided substantial controversy in the GST field. A number of transfection studies have been carried out and some disparity is apparent. The lack of drug resistance in MCF7, breast carcinoma cells transfected with GSTπ, α or μ isoenzymes (Morrow *et al.*, 1993) led many to conclude that the link between enhanced GST and drug resistance is tenuous. In other GST transfection studies only low levels of resistance (1.5- to 3-fold) were observed in the transfected cell lines (Tew *et al.*, 1993). The significance of low level resistance has been the subject of much debate. It is worth noting, in this case, that when *MDR1* recipient transfected cells are tested , they usually only display 5- to 20-fold resistance compared to the original selected *MDR1* overexpressing cell line which frequently show resistance levels of 1,000- to 10,000- fold compared to the sensitive line. For alkylating agent-resistant cell lines, the degree of selected resistance rarely exceed 20-fold; therefore it could be argued that the 1.5- to 3-fold resistance reported in the GST transfectants is relatively consistent. There have been several reports which have largely alleviated the uncertainty of the earlier data. Firstly, yeast cells, *Saccharomyces cerevisiae*, transfected with mammalian GST isoenzymes (Black *et al.*, 1990) were significantly resistant to chlorambucil and doxorubicin (maximum resistance 8- and 16-fold respectively). Secondly, in mammalian cells, Schecter *et al.* (1993) recently transfected the rat Y_C gene (α family) into rat mammary tumour cells and showed high levels of induced resistance, in the range of 6- to 30-fold, to a number of nitrogen mustards. Finally two studies have demonstrated the transfection

of Chinese hamster ovary cells with GSTπ (Miyazaki *et al.*, 1990) and GSTα (Giaccia *et al.*, 1991). GSTπ increased resistance to cisplatin and carboplatin by 2- and 3-fold, while GSTα increased protection against bleomycin. It would seem reasonable to suggest that the positive transfection studies should satisfy the question as to whether or not GSTs have a direct role in some types of tumours. However, it is important not to disregard the negative data. There are several possible explanations for this negative data: (a) intrinsic GSH/GST levels; (b) feedback control mechanisms; effective compartmentalisation of GSH/GST; (c) presence of a GSH-conjugate membrane efflux pump and (d) GSTs themselves may not be sufficient to cause resistance. In order for resistance to be observed, transfected cells may require the existence of putative cellular complimentary factors (Tew, 1994).

Increased activity of another GSH-dependent enzyme, selenium-dependent GSH peroxidase (GSPX) may augment resistance to some drugs of the classical MDR phenotype. This enzyme catalyses the elimination of some hydroperoxide toxic intermediates formed during oxidation-reduction cycling of some drugs such as the anthracyclines and mitomycin C. Overexpression of GSPX or processes which increase its substrate, reduced GSH, are potential mechanisms of antineoplastic resistance (Gessner *et al.*, 1990; Dusre *et al.*, 1990). Additionally, resistance to doxorubicin has been associated with down-regulation of NADPH reductases (enzymes implicated in the formation of toxic free radical intermediates of doxorubicin metabolism) as well as modest increases in the expression of the detoxifying enzymes, GSPX and catalase (Singh *et al.*, 1990). Finally, overexpression of glucuronyl transferase has been associated with daunorubicin resistance (Gessner *et al.*, 1990). The relevance of this observation is suggested by the ability of the enzyme to catalyse the glucuronidation and excretion of a toxic drug metabolite, daunorubinol.

There are two further mechanisms leading to resistance to alkylating agents. Firstly, increased levels of metallothioneins, proteins rich in sulphydryl-containing cysteine residues, are associated with resistance to cisplatin and alkylating agents. The mechanism is presumed to be interaction with toxic electrophilic drugs or their metabolites. Cells transfected with the human metallothionein gene (Kelley *et al.*, 1988) conferred resistance to both cisplatin and alkylating agents, suggesting a causal link between the metallothionein gene and drug resistance. However, studies of cisplatin resistant human ovarian cancer cells failed to demonstrate any link between drug resistance and metallothionein levels (Schilder *et al.*, 1990). The role of sulphydryl compounds in clinical drug resistance remains unclear. Secondly, increased expression of enzymes that increase the capacity of damaged cells to repair DNA, such as O^6-alkylguanine DNA alkyltransferase, an enzyme that removes adducts from the O^6 position of the base guanine, is associated with resistance to alkylating agents (Masuda *et al.*, 1990).

It seems unlikely that one single mechanism is responsible for the phenomenon of clinical drug resistance. It is more likely that a combination of several mechanisms including the overexpression of P-glycoprotein (or other membrane transport proteins), GSH-based detoxifying enzymes, altered topoisomerase II and DNA repair mechanism may result in resistance in the clinic.

1.3.5 Drug resistance associated with changes in topoisomerase II

(i) Introduction

Cellular control of DNA topology is achieved by the activities of the DNA topoisomerases. These enzymes modify the topological state of DNA by inducing either transient single strand (topoisomerase type I) or double strand (topoisomerase type II) breaks in DNA. The human type II enzyme is found in two forms encoded by single copy genes yeilding homodimeric proteins of 170kDa (topoisomerase IIα)

and 180kDa (topoisomerase IIβ) (Negri *et al.*, 1992). The more abundant isoform, topoisomerase IIa, is located at nuclear matrix-associated regions, a strategic location at the base of chromatin loop domains which infers both structural and functional roles (Wood *et al.*, 1990). Topoisomerase IIb is found predominantly in the nucleolus (Zini *et al.*, 1992) and binds to GC-rich sequences present in gene promotor regions, suggesting participation in gene expression (Ura *et al.*, 1991).

(ii) Topoisomerase I and drug resistance

The majority of the cell lines selected for resistance to topoisomerase I (topo I)-active agents exhibit decreased topo I activity (Nitiss *et al.*, 1988; Bjornsti *et al.*, 1989; Eng *et al.*, 1990; Sugimoto *et al.*, 1990). Selection of P388 leukaemia cells by *in vivo* treatment of tumour-bearing mice with camptothecin resulted in a cell line (P388/PC) that exhibited modest (8-fold) resistance to camptothecin (Eng *et al.*, 1990). Other cell lines selected for resistance to camptothecin-11 were also found to exhibit decreases in topo I content (Table 1.10). Selection of the P388 leukaemia cells in camptothecin-11 produced a resistant variant P388/CPT (Sugimoto *et al.*, 1990). This cell line was 45-fold resistant to camptothecin-11 yet the enzyme content was reduced by only 3-fold (Table 1.10). This indicates that other factors may also contribute to the cellular sensitivity to camptothecin-11 as well as reduced topo I levels.

In two different cell lines a second major form of resistance to camptothecin has been observed. In these cell lines the topo I is mutated and the enzyme has altered catalytic properties. Higher levels of resistance were observed in these cell lines than in the resistant cells that have a quantitative decrease in topo I activity (Table 1.10).

(iii) Topoisomerase II and drug resistance

The term 'atypical' MDR is often used to describe a pattern of MDR associated with resistance to several antineoplastic drugs including anthracyclines, epipodophyllotoxins, amsacrine (mAMSA), and mitoxantrone (Danks *et al.*, 1987).

In contrast to classical P-glycoprotein-mediated MDR, this phenotype usually retains sensitivity to the *vinca* alkaloids. The drugs associated with this resistance pattern interact with topoisomerase II and DNA in a process that leads to DNA strand breaks and cytotoxicity. This 'atypical' MDR (at-MDR) has been associated with decreased activity of type II topoisomerases (topo II) (Morrow *et al.*, 1990) and/or with the presence of variant forms of the enzyme whose activity does not facilitate drug-induced DNA damage (Zwelling *et al.*, 1989). Generally, drug accumulation is unaltered in topoisomerase-related MDR. However, a non-P-glycoprotein expressing MDR lung line has been described which showed both decreased topo II activity and reduced doxorubicin accumulation (de Jong *et al.*, 1990). Thus, multiple mechanisms of resistance may co-exist in this phenotype. Various alterations in topo II have been found in tumour cells that express the atypical-MDR (at-MDR) phenotype. These alterations are summarised in Table 1.11. In some at-MDR cell lines, resistance is related primarily to quantitative changes in topo II activity and/or protein content. In other cells that express the at-MDR phenotype, qualitative rather than quantitative alterations in target enzyme are present. In addition to these quantitative and qualitative changes, it is possible that alterations in cellular processes that follow the formation of the topo II-DNA complexes also contribute to at-MDR.

Another form of at-MDR is associated with a specific depletion of the nuclear matrix enzyme (Fernandes *et al.*, 1990). Two studies have indicated that VM-26 and m-AMSA react preferentially with the nuclear matrix DNA topo II in the parental line, CCRF-CEM leukaemia cells and induce a dissociation of newly replicated DNA from nuclear matrices of these cells (Fernandes *et al.*, 1988 & 1990). In contrast to the results obtained with the drug-sensitive CCRF-CEM cells, neither VM-26 nor m-AMSA affects the association of newly replicated DNA with nuclear matrices of the at-MDR CEM/VM-1 cells (Fernandes *et al.*, 1988). The decreased interaction of VM-26 and m-AMSA with nuclear matrix topo II of CEM/VM-1 cells is related to a selective depletion of the matrix enzyme (Fernandes *et al.*, 1990).

Table 1.10

Resistance associated with changes in topoisomerase I

CELL LINE	TOPO I ALTERATION	FOLD RESISTANCE	REFERENCE
P338/PC	Decreased topo I (2 to 4-fold)	8 (CPT)	Eng *et al.*, 1990
HT-29/CPT	Decreased topo I (8-fold)	4 (CPT-11)	Sugimoto *et al.*, 1990
St-4/CPT	Decreased topo I (4-fold)	9 (CPT-11)	Sugimoto *et al.*, 1990
P388/CPT	Decreased topo I (3-fold)	45 (CPT-11)	Sugimoto *et al.*, 1990
CPT-K5	Mutated topo I	>125 (CPT)	Tamura *et al.*, 1990
CHO/Cpt^{R}-B	Mutated topo I	>250 (CPT)	Gupta *et al.*, 1988

CPT-Camptothecin

Table 1.11

Resistance associated with changes in topoisomerase II

CELL LINE	TOPO II ALTERATION	REFERENCE
P388/AMSA (*in vivo*)	Decreased topo II activity	Tan *et al.*, 1989
KB/VP-1; KB/VP-2	Decreased topo II content	Takano *et al.*, 1991
P388/ADR/3; P388/ADR/7	Decreased topo II mRNA and activity	Deffie *et al.*, 1989
HL-60/MX/2	Decreased topo II activity; depletion of nuclear matrix topo II	Harker *et al.*, 1989
HL-60/AMSA	Point mutation on topo II gene; altered topo II catalytic activity	Hinds *et al.*, 1991
CEM/VM-1	Point mutation in topo II gene; altered topo II catalytic activity; depletion of nuclear matrix topo II	Bugg *et al.*, 1991

In summary, cellular resistance is thought to be the principle cause of treatment failure in patients undergoing therapy with anticancer agents, including topoisomerase II-directed agents. Preclinical studies with experimental anti-topoisomerase I agents suggest that the emergence of resistance will likewise limit the clinical usefulness of these drugs in many instances.

1.3.6 Regulators of programmed cell death (apoptosis) as mechanisms of drug resistance

(i) Introduction

Almost all chemotherapeutic agents ultimately affect endogenous physiological pathways for cell death to kill cancer cells. Tumour cells may become relatively more resistant either by the loss of genes required for cell death or by the over activation of genes that block it.

(ii) Definition of cell death

Cell death can be broadly defined into two mechanisms. Firstly, *necrotic* or pathologic cell death and secondly, *apoptotic* or physiologic cell death. Table 1.12 summarises the characteristics of these two types of cell death.

Knowledge about the genes involved in programmed cell death and the alterations that can occur in at least some of them in cancer has begun to lead to new strategies for the development of therapeutic agents that unlike conventional pharmaceuticals would target cell death rather than cell division.

Table 1.12

Characteristics of the mechanisms of cell death

MECHANISMS OF CELL DEATH	CHARACTERISTICS
NECROSIS (pathologic cell death)	-cellular ATP supplies are deleted owing to inability to accomplish oxidative phosphorylation -loss of osmotic equilibrium, cell swelling and rupture -spill of cellular contents to extra cellular milieu, leads to inflammatory cell response, fibrosis and scaring
APOPTOSIS (physiologic or programmed cell death)	-cell is an active participant in a process that requires energy, often depends on new RNA and protein synthesis -typically involves morphological changes: cell shrinkage, plasma membrane blebbing, nuclear fragmentation and chromatin condensation (pyknosis). -apoptotic cells are rapidly cleared through phagocytosis by viable cells

(iii) Genes involved in cell death

A variety of experimental observations have pointed to *bcl*-2 as a critical regulator of the cell death process. The *bcl*-2 (B-cell lymphoma-2) gene was first associated with the development of human neoplasia due to its frequent involvement in non-Hodgkin's lymphomas (Tsujimoto *et al.*, 1986). Though *bcl*-2 plays a major role in the pathogenesis of B-cell lymphomas, where it contributes to neoplastic cell expansion by delaying or preventing programmed cell death, perhaps more important are the potential effects of overexpression of the gene on responses to therapy. Bcl-2 protein has been shown, experimentally, to render cells more resistant to killing by dexamethasone, cyclophosphamide, doxorubicin, daunomycin, 5-fluorodeoxyuridine, 2-chlorodeoxyadenosine, fludarabine, taxol, etoposide (VP-16), camptothecin, nitrogen mustards, mitoxantrone, cisplatin, vincristine and some retinoids. The observation that Bcl-2 provides protection against such a wide variety of drugs with diverse mechanisms of action suggests that they all utilise the same final common pathway for ultimately inducing cell death and that Bcl-2 is a regulator of this pathway.

It appears that resistance to drugs mediated by the over production of Bcl-2 is distinctly different from all other mechanisms of drug resistance. For example Bcl-2 does not prevent entry of drugs into the cell or increase drug efflux out of the cell in contrast to P-glycoprotein, the product of the *MDR1* gene. Also it does not alter the extent to which drugs induce damage to DNA or the rate at which cells repair damaged DNA. In addition, Bcl-2 has no effect on nucleotide pools or rates of cell cycling. Although Bcl-2 has been reported to produce elevations in intracellular GSH levels in one cell line (Kane *et al.*, 1993), this has not been observed in several other tumour and leukaemia cell lines, indicating that there is no consistent link between Bcl-2 overexpression and increased detoxification of drug by GSH conjugation. It appears that in Bcl-2 overexpressing cells, drugs still enter the cell and induce damage, but this damage is somehow ineffectively translated into signals for cell death. In summary, Bcl-2 can convert anticancer drugs from cytotoxic to cytostatic.

(iv) Regulation of bcl-2

One mechanism that may play a role in bcl-2 gene dysregulation is the loss of function of the tumour suppressor p53. The p53 gene encodes a DNA-binding protein that functions at least in part as a transcription factor to induce cell cycle arrest and apoptosis, particularly in response to DNA damage induced by chemotherapeutic drugs and radiation (Volelstein *et al.*, 1992). The expression of the gene (*waf-1/cdi-1*) is induced by p53. This gene interferes with the function of cyclins required for G_1 to S phase transition (Marx 1993). In relation to apoptosis, it has recently been observed that gene transfer-mediated elevations in p53 activity can lead to rapid down regulation of *bcl*-2 gene expression (Miyashita *et al.*, 1994). In addition, in p53 knock-out mice, Bcl-2 protein levels were discovered to be elevated in some tissues such as the spleen , prostate and thymus. It appears that loss of p53 can lead to increases in Bcl-2 protein levels in some tissues.

It has recently been demonstrated that, in addition to transcriptional regulation of the *bcl*-2 gene by p53, it can also be regulated at the protein level by an opposing protein, Bax. This protein shares 21% amino acid identity with the Bcl-2 protein and binds to Bcl-2 preventing it from blocking cell death (Oltvai *et al.*, 1993). Loss of the *bax* gene is, therefore, equivalent to overproduction of Bcl-2. The p53 tumour suppressor can also regulate the expression of *bax* in some tissues (Miyashita *et al.*, 1994). Elevation in p53 activity leads to an up regulation in *bax* gene expression. This strengthens the connection between p53 loss in human cancers and mechanisms for dysregulation of both *bcl*-2 gene and protein function.

Although the mechanisms underlying Bcl-2 protein function and regulation of *bcl* -2 gene expression are not clearly defined, several novel treatments for cancers in which *bcl* -2 is overexpressed have been suggested. Firstly, scientists are searching by rational design and random drug screening for small molecules that could disrupt the Bcl-2/Bax interactions, thus abrogating Bcl-2 protein function. A second approach is the sequence-specific down regulation of *bcl* -2 expression. This has already been reported *in vitro* in lymphoma cell lines and acute myeloid leukaemia cells using antisense oligonucleotides (Campos *et al.*, 1994). The results from these experiments indicated that a decrease in Bcl-2 protein levels markedly enhanced sensitivity to chemotherapeutic drugs. Alternatively, certain lymphokines and retinoids, known to regulate the expression of the *bcl* -2 gene could be used to lower the levels of bcl-2 expression thus rendering *bcl* -2 overexpressing malignant cells more sensitive to the induction of apoptosis by chemotherapeutic agents and radiation. It is clear that knowledge at the molecular level of the mechanisms underlying the function of Bcl-2 and related proteins will inevitably lead to the development of novel treatments for cancer which modulate the apoptosis pathway.

Chapter 2

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2.1 Cell lines and culture conditions

A range of cultured cell lines of both human and mouse origin were used in the various studies described in this thesis. The mouse mammary carcinoma cell line EMT6/Ca /VJAC (henceforth referred to as EMT6/P) (Rockwell *et al.*, 1972) originated in a mouse alveolar tumour nodule and was successively transplanted between animal and *in vitro* culture before growth in continuous culture. The drug resistant variant of this cell line, EMT6/AR1.0 was derived by continuous step-wise *in vitro* incubation with increasing concentrations of doxorubicin (Twentyman *et al.*, 1989). These cells were finally maintained in a concentration of 1.0 μg/ml doxorubicin. EMT6 grow as attached monolayers in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with glutamine (0.5mmol/l), penicillin (100U/ml), streptomycin (100μg/ml) and 20% new-born calf serum (Sigma). They were disaggregated by rinsing the monolayers twice with a solution of trypsin (0.1%) (Life Technologies) in phosphate-buffered saline (PBS) followed by a 15 min incubation period. The cells were then resuspended in medium with mechanical disaggregation. The cell suspension was then counted manually using a haemocytometer counting chamber and then diluted appropriately for use in experiments.

NCI-H69 (henceforth referred to as H69/P) cells, originally supplied by Dr D Carney and Dr A Gazdar of the NCI/Navy Medical Oncology Branch, were derived from a patient who had received combination chemotherapy including doxorubicin. The drug resistant variant H69/LX4 was derived by continuous step-wise *in vitro* incubation with increasing concentrations of doxorubicin (Twentyman *et al.*, 1986b, Reeve *et al.*, 1989). The cells were finally maintained in a concentration of 0.4μg/ml doxorubicin. This line grows as floating aggregates of cells in RPMI 1640 medium supplemented with glutamine (0.5mmol/l), penicillin (100U/ml), streptomycin (100μg/ml) and 10% fetal calf serum (Sigma). The cells were reduced to a suspension containing small groups of cells mechanically by pipetting. In order to

count the cells accurately a small aliquot of cells was further disaggregated into a single cell suspension by passing the cells through a 21G needle and syringe. The cell suspension was then counted manually using a haemocytometer counting chamber and then diluted appropriately for use in experiments.

The T-lymphoblastoid cell line CCRF-CEM (henceforth referred to as CEM) (Foley *et al.*, 1965) and its vinblastine resistant subline CEM/VLB (Beck *et al.*, 1979) were originally obtained from Dr Rosanne Padua, University of Wales. The drug resistant variant CEM/VLB was derived by step wise *in vitro* incubation with increasing concentrations of vinblastine. These cells grow in suspension in RPMI 1640 medium supplemented with glutamine (0.5mmol/l), penicillin (100U/ml), streptomycin (100μg/ml) and 10% fetal calf serum (Sigma). The drug resistant CEM/VLB cell line is stable in culture without the addition of vinblastine. The cell suspension was counted manually using a haemocytometer counting chamber and then diluted appropriately for use in experiments.

COR-L23 (henceforth referred to as L23/P) the human large cell lung cancer cell line was derived by Ballie-Johnson *et al.* (1985). Twentyman *et al.* (1986a) derived a drug resistant variant COR-L23/R (henceforth referred to as L23/R) by step-wise incubation with increasing concentrations of doxorubicin. The cells are maintained in 0.2μg/ml doxorubicin. The L23/P and R cell lines were maintained as monolayer cultures in RPMI 1640 medium supplemented with glutamine (0.5mmol/l), penicillin (100U/ml), streptomycin (100μg/ml) and 10% fetal calf serum (Sigma). The cells were harvested by subjecting the monolayers to two rinses with a solution of PBS containing trypsin (2%) and versene (0.02%) followed by an incubation period of 15 min at 37°C. The cells were then resuspended in medium and disaggregated by pipetting. The cell suspension was counted and diluted for experimental use as previously described.

The adenocarcinoma line, MOR (henceforth referred to as MOR/P) was originally supplied by Dr M Ellison, Ludwig institute, Sutton. Like L23/R the resistant variants of MOR/P, MOR/0.2R and MOR/0.4R were derived by step-wise incubation with increasing concentrations of doxorubicin (Twentyman *et al.*, 1986a). The resistant cell lines were maintained in 0.2μg/ml and 0.4μg/ml doxorubicin respectively. These cells were maintained and prepared for experimental use under the same conditions as the L23/P and R cell lines (Twentyman *et al.*, 1986a).

All cells were maintained as stock cultures in plastic tissue culture flasks, incubated at 37°C in an atmosphere of 8% CO_2 and 92% air. Drug was removed from the resistant variants 48h prior to their experimental use. The cells were harvested in the exponential phase of growth. All cell lines are routinely screened for mycoplasma and remained negative throughout this study.

2.2 Drugs and chemicals

All Xenova compounds were dissolved in dimethylsulphoxide (DMSO) at a concentration of 5mM and stored in aliquots at -20°C. Deoxyspergualin was kindly provided by the Drug Development Branch of the National Cancer Institute, Bethesda, MD, USA. It was dissolved in sterile water at a concentration of 50mM and aliquots were stored at -20°C. Spermine and spermidine were obtained from Sigma and dissolved in sterile water at a concentration of 2M and 1.4M respectively and stored at -20°C. Cyclosporin A (Sandoz, Basel) was dissolved in absolute ethanol at 4.2mM and stored at 4°C. Cyclosporin A was routinely diluted in medium immediately before each experiment. Doxorubicin (Farmitalia) and colchicine (Sigma) were dissolved at concentrations of 0.86mM and 5mM respectively in distilled H_2O and aliquots were stored at -20°C. Taxol (Sigma) was dissolved in ethanol at a concentration of 5.8mM and stored at -20°C. Dilutions of these drugs in either PBS or culture medium was carried out immediately before

use. Calphostin C (Sigma) and staurosporine (Sigma) were dissolved in DMSO at concentrations of 0.25mM and 0.2mM respectively and stored at 4°C in the dark. H7 (Sigma) and TPA (Sigma) were dissolved in distilled H_2O at concentrations of 14.4mM and 10mM respectively and stored at 4°C in the dark. Calcein AM (Molecular Probes inc) was stored at -20°C. Probenecid (Sigma) was dissolved in DMSO at a concentration of 0.5M immediately before use.

The following tritium-labelled drugs, daunorubicin (New England Nuclear, 2.6Ci/mmol), colchicine (New England Nuclear, 60Ci/mmol) and azidopine (Amersham, 49Ci/mmol)) were stored at -70°C. [^{32}P]orthophosphate (10mCi/mg carrier free) and [^{32}P]ATP (>5000Ci/ml) were both obtained from Amersham and stored at 4°C in the dark.

Appropriate solvent controls were used in all experiments. Solvent concentrations did not exceed 0.1%.

2.3 Cytotoxicity and sensitisation assays using the tetrazolium (MTT) reduction assay

2.3.1 Background

Assays which quantitate numbers of viable cells present following therapeutic procedures have many applications in cancer research. Traditionally these assays involved counting total viable cells (using haemocytometer chambers or electronic particle counters) or colony counting (clonogenic assay). Other techniques used to assess chemosensitivity include radionucleotide incorporation assays and neutral red and trypan blue dye staining techniques. The need to process large numbers of samples led to attempts to introduce assays capable of automation. Mosmann *et al.* (1983) described a rapid colorimetric assay based on the ability of viable cells to convert a soluble tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), into an insoluble formazan precipitate (Slater *et al.*, 1963). The

purple-coloured formazan crystals may be dissolved in a variety of organic solvents and the optical density of the resulting solution measured on a multiwell spectrophotometer. The number of viable cells is proportional to the concentration of the MTT reaction product. Mosmann examined the definition of non-viable cells and concluded that only those cells with intact and functioning mitochondria could reduce MTT. The MTT dye reduction assay has several important advantages over other measures of cell viability. The assay is sensitive, reproducible over time and simple to carry out. It is adaptable to several different cell types either monolayer or suspension, cell-cell interactions are, therefore, preserved. Multiple concentrations of compound can be evaluated either alone or in combination with other agents. The effect of a compound on a number of different cell lines may also be directly compared. There are also several important limitations that should be borne in mind when using this technique. Individual cell numbers are not quantifiable and this may cause problems in interpretation of results. It is important to remember that the MTT assay is not equivalent to a clonogenic assay for a number of reasons. Whilst determining the proportion of cells with intact reproductive integrity, the MTT assay takes no account of reduced growth rate induced by a drug. It is, therefore, difficult to distinguish between cytostatic (agents producing drug induced cell cycle delay) and cytotoxic (agents causing reproductive cell death) effects from results obtained in the MTT assay. The solvent used to solubilise the formazan product is also crucial. Light scatter interference due to protein precipitation when solubilising in acid-alcohol has been reported (Ruben *et al.*, 1987). This is not a significant problem when solubilising in DMSO (Twentyman *et al.*, 1987b).

2.3.2 Method

Cytotoxicity of agents used in this study were determined using the tetrazolium (MTT) reduction colorimetric assay (Mosmann *et al.*, 1983), as adapted for use in this laboratory (Twentyman *et al.*, 1987b). Single cell suspensions were prepared

from exponentially growing cultures and inoculated into wells on 96 well tissue culture plates in a volume of 200µl/well at the following concentrations (Table 2.1):

Table 2.1

Cell plating number and assay duration

Cell line	Plating number	Assay Duration
EMT6/P	$3x10^3$	3 days
EMT6/AR1.0	$6x10^3$	3 days
H69/P	$3x10^4$	6 days
H69/LX4	$5x10^4$	6 days
CEM	$2x10^4$	4 days
CEM/VLB	$4x10^4$	4 days
L23/P	$2x10^3$	6 days
L23/R	$4x10^3$	6 days
MOR/P	$2x10^4$	6 days
MOR/0.4R	$2.5x10^4$	6 days

Cytotoxic agents were added 1 h later in a volume of 20µl. After an the appropriate incubation period (times were chosen such that untreated cells of each of the cell lines increased by 10-20 fold in number during the incubation period) 20µl of a 5mg/ml solution of MTT (Sigma) in PBS were added to each well. The plates were re-incubated for 5 h. At the end of this period the medium was aspirated from each well, 200µl of dimethylsulphoxide was added and the plates were shaken on a plate shaker for 10 min. The optical densities of the wells were read on a Titretek Multiskan MCC/340 plate reader at a wavelength of 540nm and at a reference wavelength of 690nm. For resistance modification assays the above protocol was followed with the additional step of adding the modulating agent 1 h after the cells had been plated and 1 h before the addition of the drug. In all experiments each drug

dose was studied in 4 replicate wells and variation in final absorbance between replicate wells was generally less than 10%.

2.4 Clonogenic assay

2.4.1 Background

This is an alternative method for measuring the effect of drugs or radiation on cell survival. It measures the colony-forming ability of the cells (i.e. the ability of a single surviving cell to divide and produce a colony of at least 50 'daughter' cells).

2.4.2 Method

Clonogenic survival assays were carried out on EMT6/P and EMT6/AR1.0 cells. Cells in drug-containing medium at concentrations of 50 and 200 cells/ml for EMT6/P and EMT6/AR1.0 cells respectively were plated onto 9cm Petri dishes (Nunc). They were incubated at 37°C in a humidified atmosphere of 8% CO_2 and 92% air for 9 days. At the end of this incubation period the medium was removed, the plates rinsed in saline, and then fixed and stained simultaneously in a solution of 5% crystal violet (Gurr) in 95% alcohol. Colonies were visualised with the aid of an Olympus stereo zoom microscope and those with greater than 50 cells were counted. The experiments were carried out in triplicate. After counting, the mean number of colonies per plate for each group was calculated.

Results were calculated as:

(i) Plating efficiency, given by the number of colonies on control plates calculated as a percentage of cells originally plated and

(ii) Surviving Fraction, given by the ratio of number of colonies on treated plates to number of colonies on control plates.

2.5 Drug accumulation and efflux studies

2.5.1 Measuring drug accumulation using tritium-labelled drugs

(i) Tritium-labelled drug accumulation studies in cells growing in monolayers

The ability of adherent, monolayer cells to accumulate [^{3}H]daunorubicin was determined as described previously (Wright *et al.*, 1993). Briefly, 48 hours before the experiments, cells were inoculated into 6-well plates at 2x10^4/well for EMT6/P and 4x10^4/well for EMT6/AR1.0 in a volume of 2ml. To commence experiments, medium was aspirated from the wells and replaced with medium containing [^{3}H]daunorubicin (0.1 μCi/ml) together with unlabelled drug to give a final concentration of 1μM and, where appropriate, resistance modifier (test compound) at varying concentration. As a positive control the accumulation study was also carried out in the presence of 1μM cyclosporin A. The plates were then incubated for a period of 1 h. At the end of this time, the wells were rinsed three times with ice cold PBS. One ml of distilled water was then added to each well and the plates were left for 1 h at room temperature in order to allow cell lysis to occur. At the end of this time the contents of each well were pipetted several times and 500μl was removed and placed in scintillant (Quicksafe, Zinsser Analytic). Radioactivity was measured the following day on a Beckman LS 5000CE liquid scintillation counter.

(ii) Tritium-labelled drug accumulation studies in cells growing in suspension

Cells inoculated at a concentration of 2x10^5/ml into 2ml Eppendorf tubes in a volume of 0.5ml were incubated at 37°C. After 30 min resistance modifier (test compound) at varying concentrations or cyclosporin A (2μM) were added to the appropriate tubes in a volume of 10μl. The samples were then incubated for a further 30 min before the addition of tritium-labelled cytotoxic drug to a final concentration of 0.1μCi/ml . The cells were then left to accumulate drug at 37°C for 1 h (daunorubicin) and 2 h (colchicine). After this time period the cells were rinsed three times with ice cold PBS and finally lysed in 500μl 0.1% SDS. The radioactivity was measured as described for EMT6.

(iii) Tritium-labelled drug efflux studies in cells growing in suspension

Cells inoculated at a concentration of $2x10^5$/ml into Falcon tubes in a volume of 5ml were incubated at 37°C for 30 min. The cells were loaded with tritium-labelled drug (0.1μCi/ml) in the presence of either cyclosporin A (5μM) or test compound for 30 min. Cells were then rinsed in ice cold PBS. One group of cells was resuspended in medium containing resistance modifier and the other group in medium without modifier. At each time point, beginning at 0 min, 0.5ml of cell suspension was removed from the Falcon tube and placed in a 2ml Eppendorf tube. Between time points the cells in the Falcon tubes were incubated at 37°C. The cells were pelleted by centrifugation and the medium was removed and placed in scintillation vials containing 5ml scintillant (Quicksafe, Zinsser Analytic). Radioactivity was measured as described previously.

2.5.2 Measuring drug accumulation and efflux using flow cytometry

(i) Drug accumulation

Cells inoculated at a concentration of $2x10^5$/ml into Falcon tubes in a volume of 5ml were incubated at 37°C for 30 min. After 30 min resistance modifier (test compound) at varying concentrations or cyclosporin A (2μM) were added to the appropriate tubes. The samples were then incubated for a further 30 min before the addition of fluorescent drug or dye. At each time point, beginning at 0 min, 0.5ml of cell suspension was removed from the Falcon tube and placed in a 2ml Eppendorf tube. The cells were pelleted by centrifugation and, following aspiration of the medium, cells were resuspended in 0.5ml ice cold PBS. Between time points the cells in the Falcon tubes were incubated at 37°C.

(ii) Drug efflux

Cells inoculated at a concentration of $2x10^5$/ml into Falcon tubes in a volume of 5ml were incubated at 37°C for 30 min. The cells were loaded with fluorescent drug or dye in the presence of either cyclosporin A (5μM) or test compound for 30 min.

Cells were then rinsed in ice cold PBS. One group of cells was resuspended in medium containing resistance modifier and the other group in medium without modifier. Cells were collected for sampling as in the accumulation assay.

2.6. Photoaffinity labelling of P-glycoprotein in membranes prepared from multidrug resistant cell lines

2.6.1 Isolation of plasma membranes vesicles

Membranes vesicles were prepared from the drug sensitive CEM and H69/P cells and their P-glycoprotein-overexpressing variants CEM/VLB and H69/LX4 as previously described (Barrand *et al.*, 1992). Exponentially grown cultures were washed twice in PBS containing the protease inhibitors, aprotinin (2μg/ml), leupeptin (5μg/ml) and pepstatin (0.08μg/ml). Cells growing in monolayers were harvested using a cell scraper. The cells were then pelleted at 450g for 5 min at 20°C. Following this the cells were lysed in 1mM Tris pH 7.4 containing protease inhibitors. The nuclei and unbroken cells were removed from the homogenate by centrifugation at 450g for 10 min at 4°C and the cell membranes were then separated from the resultant supernatant by centrifugation at 60000g for 1 h at 4°C.

2.6.2 Determination of protein content

The biconchininic acid (BCA) protein assay utilises the biuret reaction involving copper reacting with peptide links in proteins to yield a purple reaction product. Protein standard solutions were made up using saline solutions of bovine serum albumin (BSA) encompassing a range 40-1200μg/ml. The assay was carried out in a 96 well plate. Standards were prepared in triplicate in volumes of 10μl. PBS, 10μl, was used as a blank. Test protein samples (plus dilutions) were placed in the wells in triplicate also in a volume of 10μl. A volume of 200μl working reagent was then added to the standards, samples and blank according to the method suggested by the manufacturers. The assay was then incubated at 37°C for 30 min followed by 15 min at room temperature on a plate shaker. Spectrophotometric

quantitation of protein was then carried out at a wavelength of 540nm and at a reference wavelength of 690nm. Standard curves and sample protein content were determined using Deltasoft software package.

2.6.3 Photoaffinity Labelling

Membrane protein (50μg) in a volume of 100μl (1mM Tris HCl, pH 7.4) was incubated in 96 well plates (Falcon) for 1 h in the dark at room temperature with 0.03μM[^{3}H]azidopine (Amersham, specific activity 49 Ci/mmol) in the absence or presence of 5μM cyclosporin A or varying concentrations of resistance modifier (test compound). After incubation, samples were irradiated by placing the plates approximately 10cm away from an ultraviolet lamp (wavelength 360nm) for 30 min on ice. For control purposes one protein sample was exposed to [^{3}H]azidopine but not to UV light. The labelled membranes were then run for 45 min at 200V on a 7.5% SDS polyacrylamide gel. The gel was then fixed for 30 min in a solution of isopropanol: distilled water: acetic acid (25:65:10) and enhanced in 'Amplify' (Amersham) for a further 30 min. The enhanced gel was then dried under vacuum for 2 h and prepared for autoradiography. The fluorograms were exposed for 7 days.

2.6.4 Gel electrophoresis

For photoaffinity labelling, the labelled proteins were run on a 'Biorad' mini gel apparatus. Gels were prepared using 10ml volumes of a mixture comprising the volumes of the stock solutions shown below in table 2.2:

Table 2.2

Stock solutions for SDS polyacrylamide gels (mini gel)

GEL		STACK	
Distilled H_2O	4.9ml	Distilled H_2O	6.0ml
1.5M Tris-HCl, pH 8.8	2.5ml	0.5M Tris-HCl, pH 6.8	2.5ml
20% SDS	50µl	20% SDS	50µl
30% Acrylamide	2.5ml	30% Acrylamide	1.3ml
*10% Ammonium persulphate	50µl	*10% Ammonium persulphate	50µl
*TEMED	5µl	*TEMED	10µl

*Add immediately before pouring

TEMED is N,N,N',N'-tetramethylethylenediamine, used at 0.5% v/v.

The prepared solutions were poured between two secured glass plates and allowed to set. The glass plates containing the gel were secured in the mini gel apparatus which was then filled with electrode buffer. 5µl of sample buffer was added to 20µl of each sample before loading on to the gel. Tble 2.3 shows the recipes for the sample and electrode buffers.

Table 2.3

Recipe for sample buffer and electrode buffer

SAMPLE BUFFER		ELECTRODE BUFFER	
0.75M TRIS-HCl, pH 6.5	1ml	Tris base	9g (15g/l)
Glycerol	2ml	Glycine	43.2g (72g/l)
20% SDS	1ml	SDS	3g (5g/l)
Mercaptoethanol	1ml	Distilled H_2O	to 600ml
Bromophenol blue	a few grains		

2.7 Phosphorylation of P-glycoprotein

2.7.1 Phosphorylation of P-glycoprotein in membranes

In order to determine the effect of inhibitors and activators of protein kinase on the phosphorylation status of P-glycoprotein we adapted the method of Staats *et al.* (1990). Membranes, prepared from H69/P and H69/LX4 cells using the method outlined in section 2.5.1, were incubated in a 25µl reaction mixture as in table 2.4.

Table 2.4

Reaction mixture for phosphorylation of P-glycoprotein in membranes

Reaction Mixture	Stock Concentration	Volume (µl)
25µg protein		
0.02M Tris HCl pH 7.4	1.0M	0.5
5mM $MgCl_2$	0.1M	1.25
3mM 2-mercaptoethanol	0.1M	0.75
8µM ATP	0.9mM	2.2
3µCi [^{32}P]ATP	10µCi/µl	0.3
H_2O/Modifier		to 25µl

Incubations were carried out for 5 min on ice. The reaction was stopped by the addition of 10mM EDTA. After the addition of 5µl sample buffer the samples were electrophoresed on a 7.5% polyacrylamide gel. After electrophoresis the gel was dried on a vacuum drier for 1-2 h. Radiolabelled proteins contained in the dried gel were detected after autoradiography.

2.7.2 Gel electrophoresis

We used a large gel electrophoresis apparatus for running these samples due to the larger volumes. Gels were prepared using a mixture comprising the volumes of the stock solutions shown in table 2.5.

Table 2.5

Solutions for SDS polyacrylamide gels (large gel)

GEL		STACK	
Distilled H_2O	11.5ml	Distilled H_2O	9.2ml
1.5M Tris-HCl, pH 8.8	10.0ml	0.75M Tris-HCl, pH 6.5	3.5ml
20% SDS	200µl	20% SDS	100µl
30% Acrylamide	10.0ml	30% Acrylamide	3.0ml
*10% Ammonium persulphate	80µl	*10% Ammonium persulphate	80µl
*TEMED	50µl	*TEMED	30µl

*Add immediately before pouring

TEMED is N,N,N',N'-tetramethylethylenediamine, used at 0.5% v/v.

As with the mini gel apparatus, the prepared solutions were poured between two secured glass plates and allowed to set. The glass plates containing the gel were secured in the gel apparatus which was then filled with electrode buffer (Table 2.6). 5µl of sample buffer (see below) was added to 20µl of each sample before loading on to the gel.

Table 2.6

Recipes for sample buffer and electrode buffer

Sample buffer		Electrode buffer (10X)	
Glycerol	2ml	Glycine	145g
2-mercaptoethanol	1ml	Tris base	30g
20% Sodium dodecyl sulphate(SDS)	1ml	Sodium dodecyl sulphate(SDS)	10g
0.75M Tris HCl pH 6.5	1ml	To 1L with distilled H_2O	
Bromophenol blue	a few grains	Dilute 100ml with 900ml distilled H_2O before use to give 1X	

2.7.3 Phosphorylation and immunoprecipitation of P-glycoprotein in intact cells

In order to study the relationship between the effect of various protein kinase inhibitors on the transport of cytotoxic drugs in MDR cells and on P-glycoprotein phosphorylation, we examined P-glycoprotein phosphorylation in intact cells.

(i) Labelling

$2x10^6$ cells/ml were incubated in phosphate-free culture medium (Sigma, MEM phosphate free) containing 2% fetal calf serum for 4 h at 37°C. After this time the cells were pelleted and resuspended in fresh phosphate-free medium. The cells were then placed in 2ml aliquots in 5ml Falcon tubes before the addition of the appropriate modifier in a volume of 10µl. The cells where then re incubated at 37°C for a further 30 min before the addition of 200µCi/ml [^{32}P] orthophosphate. After addition of the label the cells were incubated for 2 h at 37°C.

(ii) Lysate preparation

The cells were pelleted by centrifugation at 450g, 4°C for 5 min and then washed with ice cold PBS(+PI) before resuspension in 2ml ice cold PBSTDS(+PI)*(see table 2.7 for recipe). The Falcon tubes were then left on ice for 10 min. After this time the cells were disrupted using a 5ml syringe and 21G needle. After initial centrifugation at 450g at 4°C for 15 min, the supernatant was transferred to 5ml Beckman tubes and centrifuged on an ultracentrifuge (Sorvall OTD 65B) at 120000g for 20 min.

Table 2.7

Recipe for PBSTDS (lysis buffer)

*PBSTDS (lysis buffer):	Quantity
10x PBS (Phosphate buffered saline)	10ml
100% Triton X-100	1ml
Sodium deoxycholate	0.5g
Sodium dodecyl sulphate (SDS)	0.1g
Distilled H_2O	to 100ml

*All solutions contain protease and phosphatase inhibitors (PI):

To 100ml:

Protease inhibitors:	200µl aprotinin (1mg/ml) 20µl pepstatin (0.4mg/ml) 500µl leupeptin (1mg/ml)
Phosphatase inhibitor:	1ml Sodium fluoride (M)

(iii) Immunoprecipitation

After preparation of the cell lysate, 1µg of C219 monoclonal antibody (stock concentration 100µg/ml) was added to 1ml supernatant in a 2ml Eppendorf tube. The tubes were placed inside a perspex box and incubated overnight at 4°C on a rocking platform. In the morning 50µl of protein A sepharose (50% v/v, Sigma) was added to each of the tubes which were then re-incubated at 4°C for a further 4 h. After this time the samples were centrifuged in a micro centrifuge at 450g, 4°C and then washed four times in ice cold PBSTDS (PI), repeating the centrifugation step between each wash. The final pellet was resuspended in 50µl sample buffer (see below), vortexed and left on ice for 30 min. The samples were then electrophoresed on a 7.5% polyacrylamide gel (see section 2.6.3 for recipes). After electrophoresis

the gel was dried on a vacuum drier for 1-2 h. Radioactively labelled proteins contained in the dried gel were detected by autoradiography.

Table 2.8

Recipe for sample buffer

Sample Buffer	
Glycerol	2ml
2-mercaptoethanol	1ml
20% Sodium dodecyl sulphate(SDS)	1ml
0.75M Tris HCl pH 6.5	1ml
Bromophenol blue	a few grains

2.8 Confocal microscopy

2.8.1 The principles of confocal imaging

The essential feature of a confocal microscope is that the illumination and detection are confined to the same spot in the specimen at any one time. If the spot is so small that its limits are set by diffraction, the resolution in a confocal microscope is greater than in a conventional one. The key feature of confocal imaging is that only objects or sections of objects that are in focus are detected. Out of focus regions appear black and therefore do not contribute to the image. A confocal microscope does not produce a complete optical image of the specimen. To build up an image it is necessary to scan the point probe over the field of view. This is done, in the case of the MRC 600 model, by scanning the beam over a fixed specimen (beam scanning). The image is generated electronically from a serial signal derived from the photomultiplier output. The MRC 600 confocal microscope used in the laboratories in Cambridge is of the epi-illumination design. In this design the same lens functions as both condenser and objective. This eliminates the need for exact matching and co-orientation of two lenses. Light from an aperture is reflected into

the rear of the objective lens and is focused on the specimen. Light returning from the specimen, as a result of either reflection or fluorescence, passes back through the lens and is focused on a second aperture, which allows a portion of the beam to pass to a detector such as a photomultiplier. The MRC 600, manufactured by Biorad, was developed from the MRC 500 confocal microscope originally developed in the Medical Research Council laboratories in Cambridge.

2.8.2 Method

(i) For cells growing in suspension:

CEM and CEM/VLB cells were suspended at a concentration of $2x10^5$ cells per ml in a 5ml Falcon tube and incubated at 37°C. Test XR compound was added to the cell suspension at a concentration of 5μM. After 30 min 10μM doxorubicin was added to the cells which were then incubated at 37°C for a further 2 h. The Falcon tubes were then centrifuged at 450g for 5 min at 4°C. The medium was then carefully aspirated and the cells were resuspended in 100μl of ice cold PBS. Approximately 20μl of this cell suspension was then placed onto a cover slip using a Gilson pipette. The cover slip was then inverted with the cell layer face down onto a clean glass microscope slide and sealed around the edges with clear nail varnish to prevent drying out of the preparation.

(ii) For cells growing as attached monolayers:

L23/P and L23/R cells were seeded in a volume of 2ml and at a concentration of $5x10^4$/ml into 6 well plates containing a sterile coverslip in each well. After 48h test XR compound was added to each well to a concentration of 5μM. After 30 min 10μM doxorubicin was added to the well to produce a concentration of 10μM. The plates were then incubated at 37°C for a further 2h. After this time the cover slips were removed from the wells and carefully rinsed twice in PBS by gripping the cover slip on the edge with a pair of tweezers and dipping it into two beakers of ice cold PBS. The excess PBS was then allowed to drain off before the cover slip was

inverted with the cell layer face down onto a clean glass microscope slide and sealed around the edges with clear nail varnish.

2.9 Glutathione (GSH) Assays

2.9.1 Intracellular GSH levels

Cells at a concentration of 2-4x10^6/ml were suspended in medium and placed in 2ml Eppendorf tubes (1ml per tube). Test drugs or solvent were then added to the cells and the tubes were incubated at 37°C for 1 h. After this time the cells were pelleted in a microfuge and rinsed twice in ice cold PBS. The pellet was the resuspended in 200µl of solution A (EDTA 100µl : distilled H_2O 80µl :TCA 20µl). The tubes were then vortexed and left on ice for 15 min. before being vortexed again. Finally, the tubes were centrifuged at maximum speed in a microfuge for 5 min. The supernatant is used for GSH determination. GSH levels were determined using a colorimetric assay. carried out in a 96 well plate (see table 2.9 for format). Sample GSH levels are determined from a standard curve. The colorimetric assay used here is based on the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by SH groups to form 2-nitrobenzoic-5-mercaptobenzoic acid. The nitromercaptobenzoic acid anion has an intense yellow colour and can be used to measure SH groups and, therefore, GSH.

Table 2.9

Format for GSH determination colorimetric assay

	1 Standard*	2	3	4 S	5 A	6 M	7 P	8 L	9 E	10 S	11	12 Blank
A	0 (67)											blank
B	10 (57)											blank
C	20 (47)											blank
D	30 (37)											blank
E	40 (27)											blank
F	50 (17)											blank
G	67 (0)											blank
H	-											blank

* Values are volume of GSH standard in µl. Parentheses indicate volume of EDTA in µl

Each well contained: 133µl Tris buffer (pH8.9), 67µl of either sample (in sample columns 2-11), GSH standard/EDTA mix (in standard column 1) or EDTA (in blank column 12), 3.3µl DTNB.

After the addition of all the reagents, the plate was placed on a plate shaker for 15 min. The optical densities of the wells were then read on a Titertek Multiskan MCC/340 plate reader at a wavelength of 412nm.

2.9.2 Cellular GSH release

Cells were suspended in 1ml of medium at a concentration of 5-10x10^6/ml in Falcon tubes. Test drugs or solvent were then added to the cells and the tubes were incubated at 37°C for 1 h. After this time the cells were pelleted and rinsed twice in ice cold PBS. The pellet was then resuspended in 100µl Kreb's buffer (+1µl acivicin 20mM- a γ-glutamyl transpeptidase (GGT) inhibitor) in 2ml Eppendorf tubes (each sample group was evaluated in duplicate). The cells were then incubated for a further 1 h at 37°C. After this time the cells were again pelleted and

the supernatant was used for GSH measurement. To allow GSH release over 1 h to be calculated the experiment was carried out in duplicate. On addition of the Kreb's buffer, one of the duplicate sets of Eppendorf (i.e 2 out of 4) tubes were spun immediately to pellet the cells and remove the supernatant. GSH release prior to the 1 h incubation was also, therefore, determined. The colorimetric assay was carried out in a 96 well plate. as in table 2.9. The standard column was, however, different (Table 2.10).

Table 2.10

GSH standards for GSH release assay

500µM GSH stock (volume µl)	Volume EDTA (µl)	GSH (nM)
0	60	0
10	50	5
20	40	10
30	30	15
40	20	20
50	10	25
60	0	30

GSH release was calculated as nmoles GSH per 10^6 cells per h by the following equations:

(1) Absorbance (sample) / Absorbance (1nM GSH- from standard curve)

(gives nmoles GSH in 60µl sample)

(2) (1) x 0.1 (ml) / 0.06 (ml)

(gives nmoles GSH in whole sample)

(3) (2) x10^6 / total number of cells

(gives nmoles GSH/10^6 cells/h)

Chapter 3

The effect of deoxyspergualin on multidrug resistant cells

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3.3.5 Inhibition of [^{3}H]Azidopine covalent binding to P-glycoprotein in membranes prepared from H69/LX4 cells.

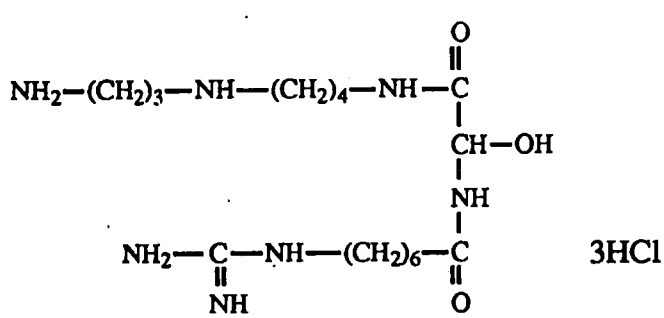
3.4 Discussion

3.1 Introduction

Deoxyspergualin (Figure 3.1) is a synthetic derivative of the anti-tumour antibiotic spergualin produced by a strain of *Bacillus laterosporus* (Takeuchi *et al.*, 1981; Umezawa *et al.*,. 1981). Spergualin has good anti-tumour activity against murine neoplasms, including the lymphatic leukaemias L1210 and P388, mastocytoma P815, thymoma EL-4, sarcoma 180, and the myeloid leukaemia 1498 (Takeuchi *et al.*, 1981). Deoxyspergualin, currently in the National Cancer Institute (NCI) decision network, is more potent *in vivo* than spergualin and its direct synthesis is simpler therefore it was selected as a candidate for further development. In addition to its antitumour activity, deoxyspergualin has also been shown to possess immunosuppressive activity in both *in vitro* and *in vivo* systems (Nemoto *et al.*,. 1987; Shiro *et al.*, 1992). The mechanism of immunosuppression of the drug is at present unclear, however, deoxyspergualin has been shown to increase interleukin 2 production in mixed lymphocyte culture, to enhance natural killer cell activity in spleen cells of tumour bearing mice, and to activate T-lymphocytes (Ishizuka *et al.*, 1986).

Figure 3.1

The structure of deoxyspergualin



Cyclosporin A and FK506 are also natural products possessing potent immunosuppressive properties (Shiro *et al.*, 1992). Deoxyspergualin has been shown to bind to the human constitutive heat shock protein Hsp70 (Nadler *et al.*,

1992). Cyclophilin, the cyclosporin A binding protein, FK506 binding protein, and the heat shock proteins are all involved in the regulation of protein folding. This and the ability of all three agents to inhibit the function of T-cell lymphocytes may suggest a common mechanism of action for these immunosuppressants.

Cells treated *in vitro* with one of a well defined group of, mainly natural product, cytotoxic agents acquire resistance to that agent and become simultaneously resistant to many other drugs that are structurally and functionally unrelated to the selecting drug (Biedler *et al.*, 1970; Endicott *et al.*, 1989). Drugs involved include doxorubicin, vincristine, etoposide and taxol. These cells are said to possess a multidrug resistant phenotype and frequently overexpress a 170-kDa membrane glycoprotein (P-glycoprotein) which is believed to act as a drug efflux pump (Endicott *et al.*, 1989). This type of resistance is often referred to as 'classical MDR'.

Both FK506 and cyclosporin A have been shown to act as effective modifiers of classical MDR in that they can selectively restore sensitivity to the cells with acquired resistance (Jachez *et al.*, 1993; Twentyman *et al.*, 1987a). Deoxyspergualin is similar to cyclosporin A and FK506 in its properties and mechanism of action and multidrug resistant cells are cross resistant to cyclosporin A. We decided therefore to address the questions, does deoxyspergualin act as a modifier of P-glycoprotein-mediated MDR and are multidrug resistant cells cross resistant to deoxyspergualin?

The structure of the polyamine spermidine is present within the larger structure of deoxyspergualin, which can therefore be considered to be a spermidine analogue. Bovine serum copper amine oxidase catalyses the oxidative deamination of spermidine to produce an aminoaldehyde, ammonia and hydrogen peroxide. It is thought that these aminoaldehydes are responsible for the toxicity of polyamines *in vitro* in the presence of bovine serum (Kunimoto *et al.*, 1985; Parchment *et al.*,

1990) whereas other mechanisms may predominate in this cytotoxicity under conditions where other serum types, low in copper amine oxidase content, are used. For this reason all experiments involving the incubation of cells in medium were performed in duplicate using both bovine serum and horse serum, which is low in copper amine oxidase content (Kuramochi *et al.*, 1987).

3.2 Materials and methods

3.2.1 Cell lines

We used the mouse mammary carcinoma cell line EMT6/P and the human small cell lung cancer cell line H69/P together with their P-glycoprotein overexpressing sublines EMT6/AR1.0 and H69/LX4. For cell culture conditions see Section 2.1. All experiments involving the incubation of cells in medium were performed in duplicate using both bovine serum and horse serum.

3.2.2 Drug sensitivity testing

The cytotoxicty of deoxyspergualin and the polyamines, spermidine and spermine were determined using the tetrazolium (MTT) reduction colorimetric assay. Clonogenic survival assays were also carried out on EMT6/P and EMT6/AR1.0 cells exposed to increasing concentrations of deoxyspergualin. The details of both these assays are described in Sections 2.3 and 2.4.

3.2.3 Drug accumulation studies

The effect of deoxyspergualin on the ability of EMT6/P and EMT6/AR1.0 cells and H69/P and H69/LX4 cells to accumulate [^{3}H]daunorubicin was determined as described in Section 2.5.2.i and 2.5.2.ii respectively.

3.2.4 Photoaffinity labelling

Membranes were prepared from H69/P and H69/LX4 cells as described in Section 2.6.1. Membrane protein (50μg) in a volume of 100μl (1mM Tris HCl pH 7.4) was incubated for 1 h in the dark at room temperature with 0.03μM[^{3}H]azidopine (Amersham, specific activity 49 Ci/mmol) in the absence or presence of 5μM cyclosporin A or increasing concentrations of deoxyspergualin (1-100μM). The details of this assay are described in Sections 2.6.

3.3 Results

3.3.1 Toxicity of polyamines and deoxyspergualin

Both parent and resistant cell lines were more sensitive to the polyamines spermidine and spermine in the presence of bovine serum compared to horse serum. Typical data sets are shown in Figures 3.2. & 3.3. In the presence of bovine serum, EMT6/P, EMT6/AR1.0, H69/P and H69/LX4 cells are respectively 32, 32, 372 and 483 fold more sensitive to spermidine than in the presence of horse serum. Corresponding figures for spermine are 175, 133, 321 and 444 fold respectively (Table 3.1). Interestingly in the human cell lines, H69/P and H69/LX4 the ratios of IC_{50}s in bovine serum compared to horse serum for spermine and spermidine are respectively 2-3 and 10-15 fold greater than in the mouse cell line EMT6/P and EMT6/AR1.0. This may be due to the differing levels of copper amine oxidase present in the two types of bovine serum (new-born calf serum in EMT6 and foetal calf serum in H69) used to culture the different cell lines.

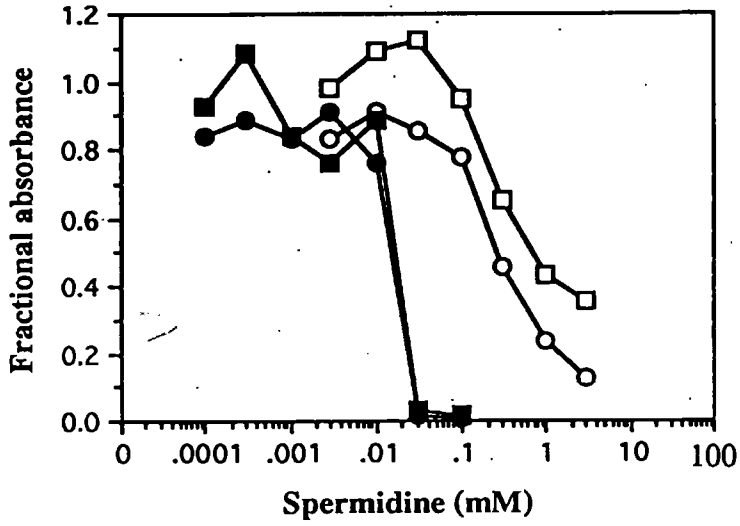
Deoxyspergualin is an analogue of spermidine. However, its relative toxic effects in the different serum types do not reflect those of the polyamines in the different serum types. When fractional absorbance is plotted against increasing drug concentration (Figure 3.4), experiments carried out in the presence of bovine serum result in much steeper curves than those obtained in the presence of horse serum. The surviving fraction in horse serum supplemented medium typically falls from 80% to 20% between 1 and 100 μM deoxyspergualin. In the bovine serum supplemented medium a similar fall in the curves is produced by only a 2-5 fold dose increase. Similar results were obtained in a number of independent experiments.

EMT6/AR1.0 and H69/LX4 show modest cross resistance to deoxyspergualin, spermidine and spermine in both bovine serum and horse serum although this is only significant ($p<0.05$, Student's t test) in EMT6/AR1.0 for deoxyspergualin in bovine serum and horse serum and for spermidine in horse serum alone (Table 3.2).

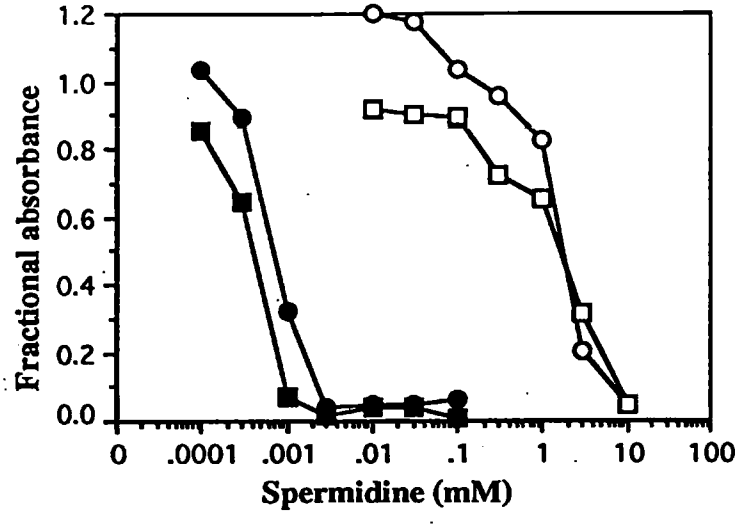
Figure 3.2

The toxicity of spermidine in (a) EMT6/P and EMT6/AR1.0 and (b) H69/P and H69/LX4 in the presence of horse serum and bovine serum.

(a)



(b)

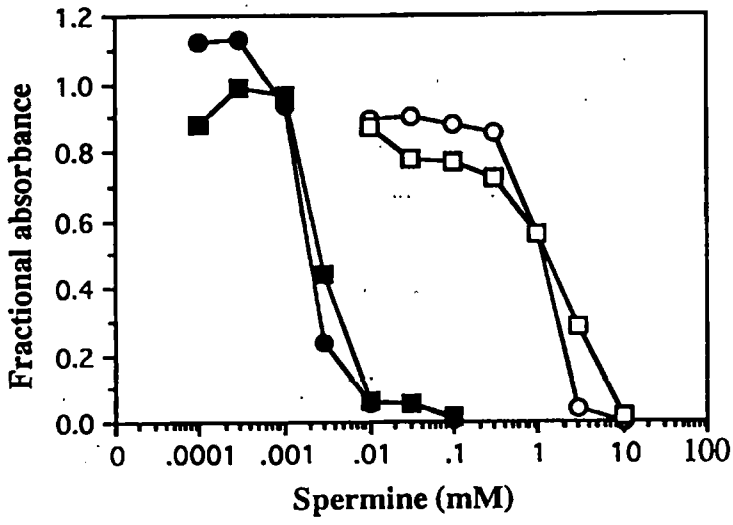


(●) parent, bovine serum, (○) parent, horse serum, (■) resistant, bovine serum, (□) resistant, horse serum. Figures represent typical data sets. Similar results were obtained in a number of independent experiments. Fractional absorbance is defined by mean optical density of treated group divided by that of the control group

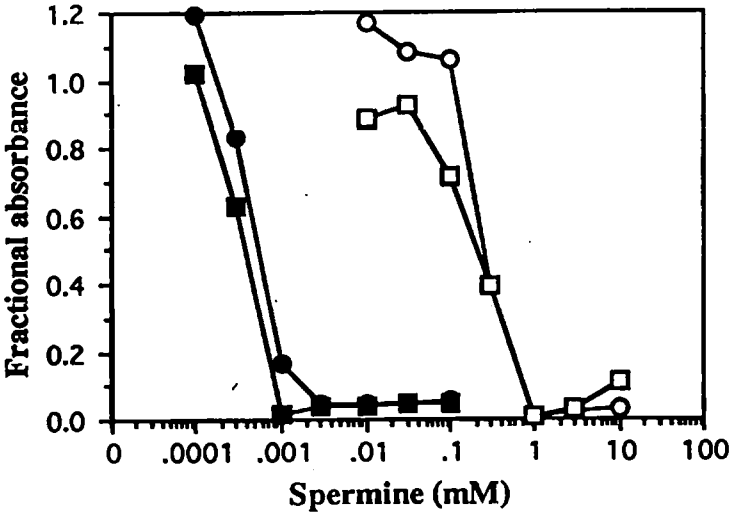
Figure 3.3

The toxicity of spermine in (a) EMT6/P and EMT6/AR1.0 and (b) H69/P and H69/LX4 in the presence of horse serum and bovine serum.

(a)



(b)

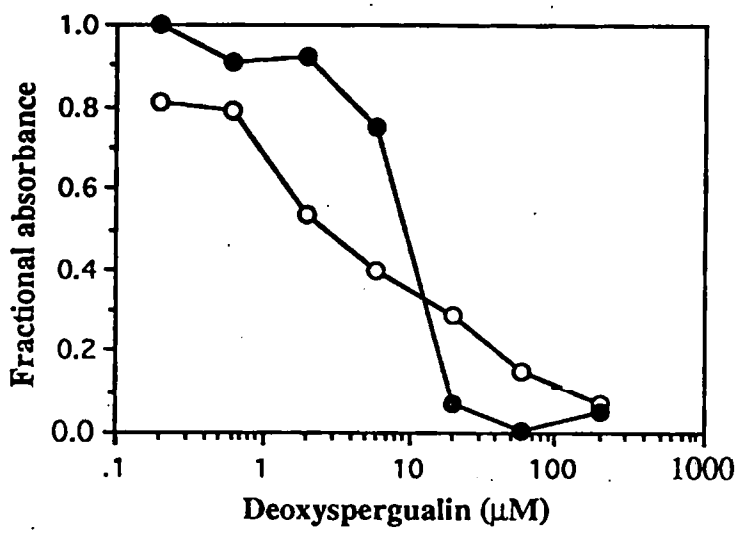


(●) parent, bovine serum, (○) parent, horse serum, (■) resistant, bovine serum, (□) resistant, horse serum. Figures represent typical data sets. Similar results were obtained in a number of independent experiments. Fractional absorbance is defined by mean optical density of treated group divided by that of the control group

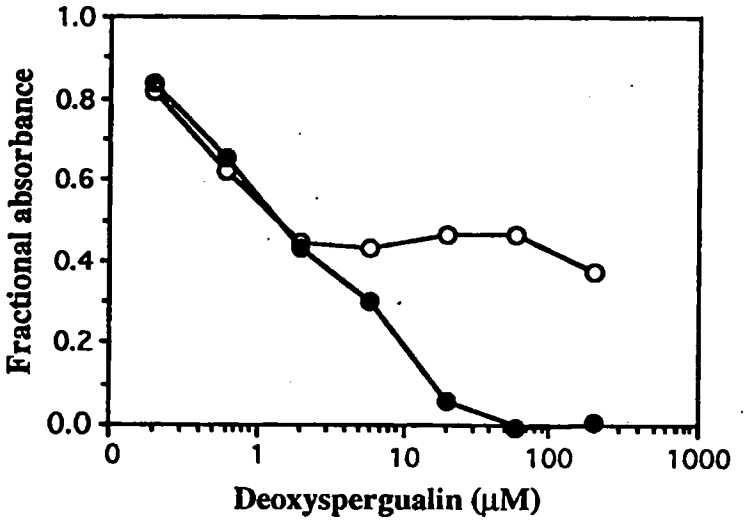
Figure 3.4

The toxicity of deoxyspergualin

(a) EMT6/P



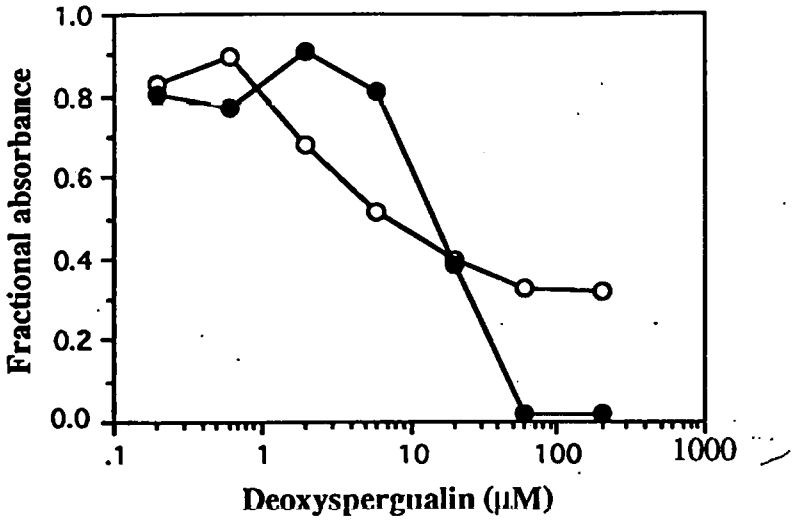
(b) H69/P



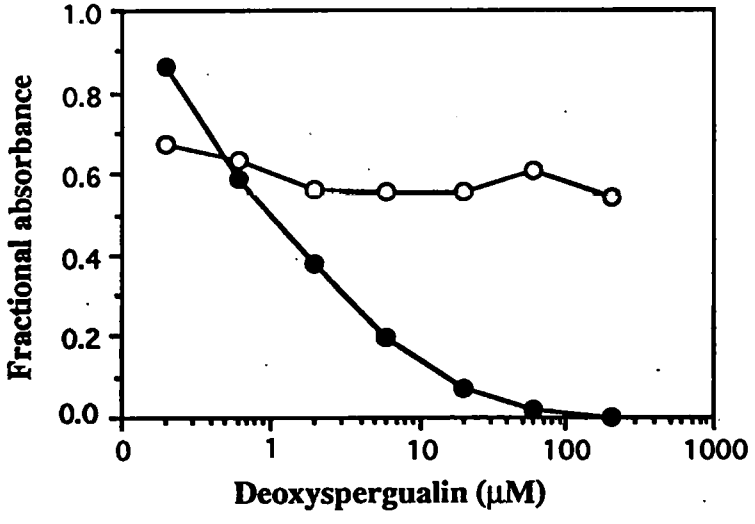
bovine serum (●) and horse serum (○)

Figure represent typical data sets. Similar results were obtained in a number of independent experiments. Fractional absorbance is defined by mean optical density of treated group divided by that of the control group

(c) EMT6/AR1.0



(d) H69/LX4



bovine serum (●) and horse serum (○)

Figure represent typical data sets. Similar results were obtained in a number of independent experiments. Fractional absorbance is defined by mean optical density of treated group divided by that of the control group.

Table 3.1

Ratio of IC_{50}[a] in bovine serum compared to horse serum

	EMT6/P	EMT6/AR1.0	H69/P	H69/LX4
Spermine	200 150 n=2	133 133 n=2	320 (68) n=4	443 (33) n=4
Spermidine	31[b] (7) n=3	31 (10) n=3	372 (39) n=3	483 (17) n=3

[a]IC_{50}=concentration of drug required to reduce fractional absorbance to 50% of control value

[b]Values shown are means (standard error) from n independent experiments.

Where n=2, individual values are shown

Table 3.2

Resistance factors[a] for spermine, spermidine and deoxyspergualin

	Spermine		Spermidine		Deoxyspergualin	
RATIO	BS	HS	BS	HS	BS	HS
EMT6/AR1.0 / EMT6/P	1.7[b] (0.2) n=4	2.2 (1.2) n=4	2.3 (1.5) n=4	1.9[c] (0.5) n=4	1.9[c] (0.7) n=6	2.4[c] (0.4) n=6
H69/LX4 / H69/P	1.3 (0.6) n=6	1.7 (1.2) n=6	1.2 (0.9) n=4	1.4 (0.5) n=4	1.2 (0.2) n=6	1.8 (0.5) n=6

[a]Resistance factor is defined by the IC_{50} of drug in resistant line

divided by the IC_{50} of drug in the sensitive line

[b]Values are means (standard error) from n independent experiments

[c]$p<0.05$, value is significantly different from that of the control (Student's t test)

3.3.2 Effect of continuous exposure of deoxyspergualin on the colony forming potential of EMT6/P and EMT6/AR1.0

To investigate further the differing response of EMT6/P and EMT6/AR1.0 to deoxyspergualin in the different serum types we employed the clonogenic assay to examine the effect of deoxyspergualin (continuous exposure) on the number and size of colonies formed. The results obtained in the presence of bovine serum were similar to those produced in the MTT assay. The surviving fraction of colonies fell from 80% to 20 % between 2 and 20μM deoxyspergualin (Figure 3.5). However, in the presence of horse serum the curve dropped more steeply than the curve produced in the MTT assay. This is likely to be due to the fact that in horse serum, as deoxyspergualin concentration increased, colonies reduced in size (i.e. number of cells per colony) rather than number. Only those colonies with 50 or more cells are included in the count. At higher doses of deoxyspergualin (>0.1μM), there were many colonies containing fewer than 50 cells which were therefore not included in the count. This accounts for the steeper curve obtained in this assay compared to the MTT assay which is contributed to by all viable cells without an arbitary cut-off point. These data indicate that deoxyspergualin is cytostatic to EMT6/P and EMT6/AR1.0 in the presence of horse serum and cytotoxic in the presence of new-born calf serum.

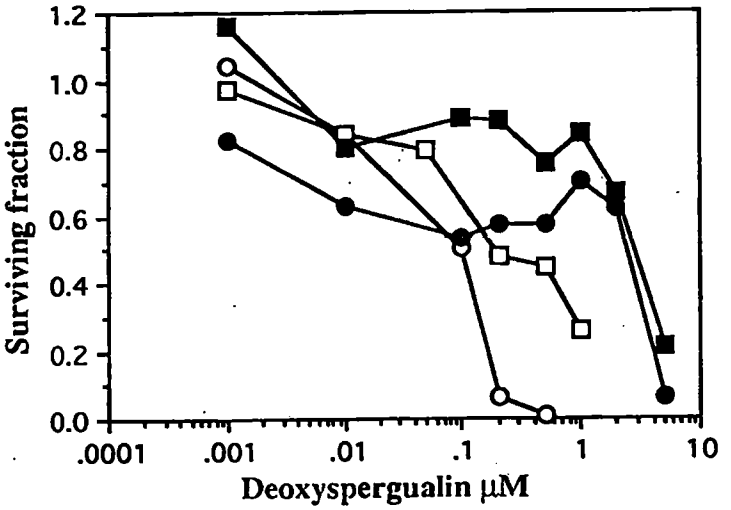
3.3.3 Effect of deoxyspergualin on the accumulation of [^{3}H]daunorubicin in EMT6/AR1.0 and H69/LX4 cells

There was no difference in the accumulation of [^{3}H]daunorubicin between experiments carried out using medium supplemented with either 10% bovine serum or 10% horse serum in either the parent or resistant cell line. Figures 3.6.a & b show that deoxyspergualin, at concentrations of up to 200μM did not reverse the accumulation deficit in EMT6/AR1.0 or H69/LX4 cells. In comparison, cyclosporin A (1μM) had an accumulation ratio of 17.6 in EMT6/AR1.0 (where accumulation ratio is defined by drug uptake in the presence of modifier divided by drug uptake

in the absence of modifier) and restored [^{3}H]daunorubicin accumulation to levels comparable to the parent line. The differential in accumulation between parent and resistant cells is much lower in the human cell line than the mouse cell line. Cyclosporin A (1.0μM) did not reverse the accumulation deficit in LX4 cells to the same extent as in EMT6/AR1.0 cells. However, cyclosporin A (5.0μM) restored [^{3}H]daunorubicin accumulation to levels higher than those of the parent line.

Figure 3.5

Effect of deoxyspergualin on the colony forming ability of EMT6/P and EMT6/AR1.0 in the presence of either horse serum or bovine serum

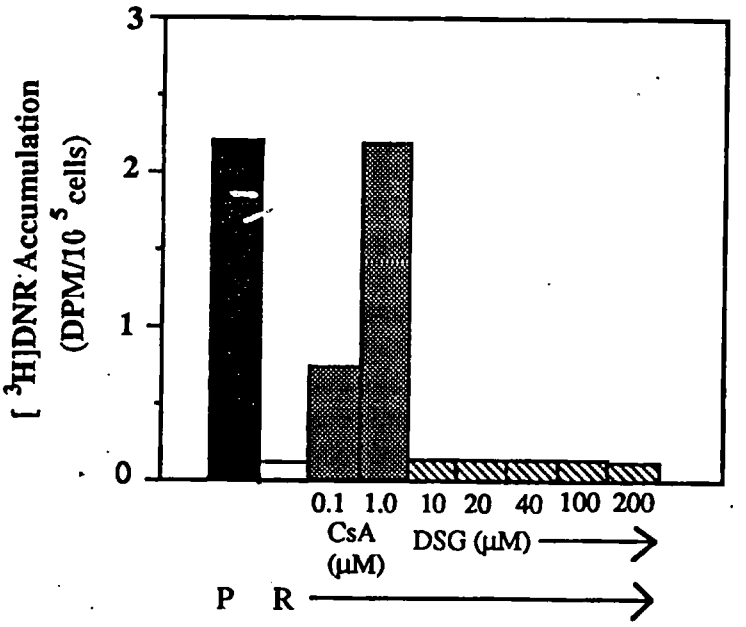


horse serum EMT6/P (○), EMT6/AR1.0 (□)) or bovine serum (EMT6/P (●), EMT6/AR1.0 (■)

Figure represent typical data sets. Similar results were obtained in 2 independent experiments.

Figure 3.6

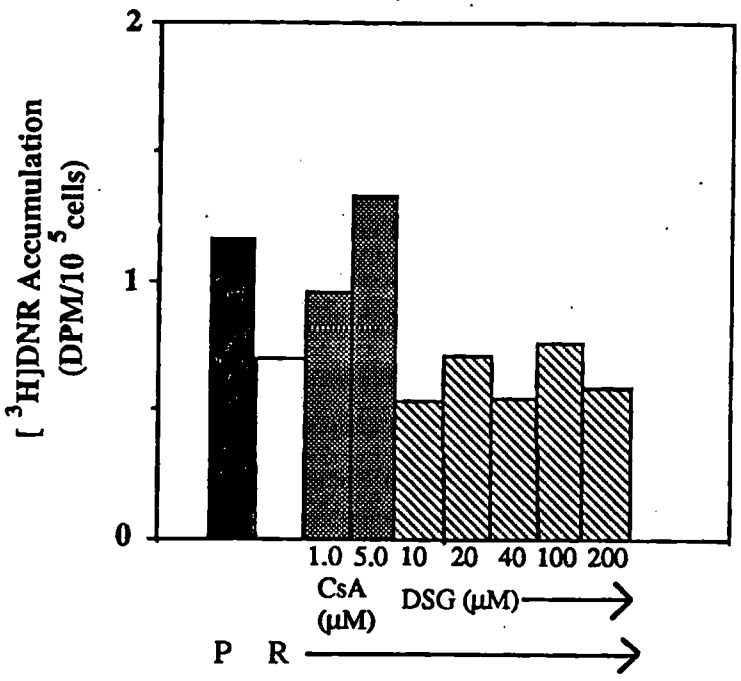
(a) The effect of cyclosporin A (CsA) and deoxyspergualin (DSG) on the accumulation of [^{3}H]daunorubicin (DNR) in EMT6/P (P) and EMT6/AR1.0 (R) cells.



Parental cell line (■), Resistant cell line (□), Resistant cell line + cyclosporin A (▩), Resistant cell line + deoxyspergualin (▨). Figures represent typical data sets. Similar results were obtained in a number of independent experiments.

Figure 3.6

(b) The effect of cyclosporin A (CsA) and deoxyspergualin (DSG) on the accumulation of [^{3}H]daunorubicin (DNR) in H69/P (P) and H69/LX4 (R) cells.



Parental cell line (■), Resistant cell line (□), Resistant cell line + cyclosporin A (▩), Resistant cell line + deoxyspergualin (▨). Figures represent typical data sets. Similar results were obtained in a number of independent experiments.

3.3.4. Effect of deoxyspergualin on the sensitivity of EMT6/AR1.0 and H69/LX4 cells to doxorubicin.

There was no significant difference between the IC_{50}s of EMT6/AR1.0 in the presence or absence of deoxyspergualin in either serum type. However, deoxyspergualin (4μM) caused a modest but significant decrease in IC_{50} for doxorubicin in H69/LX4 in the presence of horse serum ($p<0.05$, Student t test). In the presence of bovine serum there was no effect (Table 3.3).

Table 3.3

Effect of deoxyspergualin on the IC_{50}[a] of doxorubicin in EMT6/AR1.0 and H69/LX4

	EMT6/AR1.0 (IC_{50} μM)		H69/LX4 (IC_{50} μM)	
	BS	HS	BS	HS
Control	7.9[b] (2.6)	4.0 (2.4)	1.1 (0.5)	1.3 (0.5)
Deoxyspergualin (2μM)	8.0 (2.6)	7.5 (3.7)	1.5 (0.8)	1.8 (0.4)
Deoxyspergualin (4μM)	6.2 (1.0)	7.8 (4.5)	1.7 (0.9)	1.6[c] (0.5)

[a]IC_{50}=dose of drug required to reduce fractional absorbance to 50% of control value

[b]Values are means (standard error) from at least 3 independent experiments

[c]$p<0.05$, value is significantly different from that of the control (Student's t test)

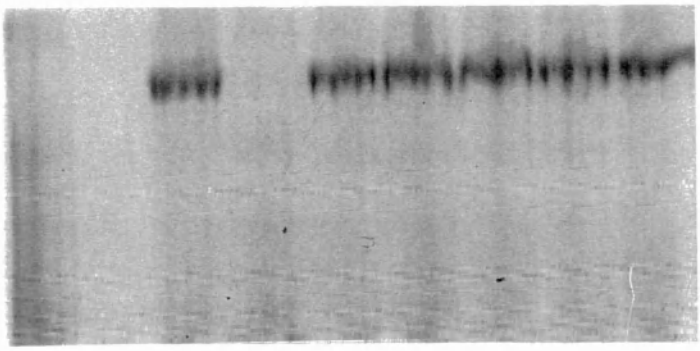
3.3.5 Inhibition of [^{3}H]Azidopine covalent binding to P-glycoprotein in membranes prepared from H69/LX4 cells.

The potential of deoxyspergualin to inhibit the covalent binding of [^{3}H]azidopine to P-glycoprotein was studied using the photoaffinity labelling technique. Figure 3.7 shows that the covalent binding of [^{3}H]azidopine is only seen after exposure to UV

irradiation. With UV exposure there is a clear band at a molecular weight of 170kDa in H69/LX4 but not in H69/P indicating the presence of [^{3}H]azidopine binding to P-glycoprotein. Covalent binding of [^{3}H]azidopine to P-glycoprotein only occurs in the resistant cell line after UV irradiation. Deoxyspergualin at concentrations of between 2 and 40 μM does not inhibit the covalent binding of [^{3}H]azidopine to P-glycoprotein. In contrast, however, clear inhibition was produced by cyclosporin A (1μM).

Figure 3.7

Effect of deoxyspergualin (DSG) on the covalent photo incorporation of [^{3}H]azidopine to P-glycoprotein.



Lane number **1** **2** **3** **4** **5** **6** **7** **8** **9**

Lane 1: H69/P membranes, **Lanes 2-9:** H69/LX4 membranes . (Lane 2: no UV exposure, Lane 3: no modifier, Lane 4: + CsA 5μM, Lane 5, 6, 7, 8 and 9: + DSG 2, 4, 10, 20 and 40μM respectively.)

Figure represent typical data sets. Similar results were obtained in 3 independent experiments.

3.4 Discussion

Copper amine oxidase contained in serum is thought to be responsible for the cytotoxicity of polyamines and deoxyspergualin *in vitro* (Shiro *et al.*,. 1992). Our studies support the theory that aminoaldehydes and hydrogen peroxide produced by the oxidative deamination of polyamines by copper amine oxidases are responsible for the cytotoxicity of these agents *in vitro* in the presence of bovine serum. However our results using horse serum which is low in copper amine oxidase content indicate that this may not be the only mechanism of toxicity. Spergualin has potent anti-tumour activity in mouse neoplasms (Takeuchi *et al.*, 1981). Mouse serum is poor in copper amine oxidase (Shiro *et al.*, 1992), therefore the antitumour effect of deoxyspergualin is unlikely to be due to its oxidation in murine blood. Hence the mechanism of cytotoxicity in this system may be other than via oxidative deamination by copper amine oxidase. The antitumour activity of spergualin was markedly reduced in immuno-deficient mice (Masaaki *et* al., 1986). It may therefore be possible that *in vivo* in the presence of serum types which are low in copper amine oxidase, e.g. mouse and human serum, the anti-tumour activity of deoxyspergualin may be due to activation of the immune system.

Kuramochi *et al.* (1987) also showed that the antiproliferative action of deoxyspergualin is different to that induced by copper amine oxidase. This group demonstrated that aminoguanidine, a potent inhibitor of copper amine oxidase activity did not decrease the growth inhibitory activity of deoxyspergualin in the presence of human serum, which is low in copper amine oxidase activity. They also showed that the survival curves produced by exposure of cells to deoxyspergualin in the presence of human serum were shallow and therefore concluded that this may be due to the mechanism of action of deoxyspergualin in the presence of human serum being cytostatic rather than cytocidal. Our survival curves produced by plotting surviving fraction, determined by measuring fractional absorbance in the MTT assay, against $\log_{10}$ (concentration of drug) are also shallow in the presence of

horse serum. Our results therefore support those of Kuramochi *et al.* and it appears that in the absence of copper amine oxidase the mechanism of action of deoxyspergualin may indeed be cytostatic.

Nishikawa *et al.*, (1991) showed that deoxyspergualin arrested cells in G_0/G_1 phase and reduced the cycling cell population. The conversion of G_0 to G_1 phase and progression to S phase are two critical steps for cell proliferation. Our results show that in the absence of copper amine oxidase present in bovine serum, deoxyspergualin appears to exert a cytostatic effect on the cell population reflecting the drugs effect on cell cycle.

Cyclosporin A and FK506 are natural products possessing potent immunosuppressive properties and also the ability selectively to restore sensitivity to cells with acquired MDR. By studying the uptake of radio-labelled daunorubicin we were able to show that deoxyspergualin, unlike cyclosporin A and FK506, did not alter the drug accumulation deficit present in the cell line EMT6/AR1.0 and H69/LX4.

The use of the photo-active, tritium-labelled, arylazide, azidopine has enabled us to demonstrate that resistance modifiers such as cyclosporin A are able to inhibit the covalent binding of [^{3}H]azidopine to P-glycoprotein. The results obtained from our photoaffinity labelling experiments here show that deoxyspergualin does not inhibit the covalent binding of azidopine to P-glycoprotein suggesting that it is not a substrate for the protein. This is supported by the fact that deoxyspergualin does not reverse the accumulation deficit in EMT6/AR1.0 or H69/LX4 and that it is not capable of restoring the sensitivity of the drug resistant cell lines EMT6/AR1.0 or H69/LX4 to doxorubicin to the levels of the parent cells indicating deoxyspergualin does not modify classical MDR.

The P-glycoprotein overexpressing human, MDR variant, H69/LX4 shows no cross resistance resistance to deoxyspergualin. The mouse EMT6/AR1.0 cells however, show modest but significant cross resistance to the drug. Deoxyspergualin is not a substrate for human P-glycoprotein, as we demonstrated using photoaffinity labelling, and therefore is not removed from the human cells by the active transport drug efflux pump. It is possible that deoxyspergualin has a low but significant affinity for mouse P-glycoprotein, however the cross resistance observed in the mouse line may be due to an alternative mechanism other than incresed efflux by P-glycoprotein.

Cyclosporin A binds to a cytosolic protein, cyclophilin. FK506 also binds to an intracellular binding protein. Both these proteins have been shown to possess peptidyl-propyl cis-trans isomerase activity. If Cyclosporin A and FK506 exert there resistance modifying activity, at least in part, by the inhibition of peptidyl-propyl cis-trans isomerase activity then this may account for the lack of activity of deoxyspergualin which does not inhibit this enzyme (Nadler *et al.*, 1992).

Our data suggests that deoxyspergualin, unlike Cyclosporin A and FK506, is not a good candidate for development as a resistance modifier. However, its maintenance of activity in classical multidrug resistant cells and its potent *in vivo* anti-tumour activity makes deoxyspergualin a promising agent for further investigations into its potential clinical use as an anti-tumour agent.

Chapter 4

The activity of novel fungal product diketopiperazines as modifiers of P-glycoprotein-mediated multidrug resistance

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4.4.4 Summary

4.1 Introduction

4.1.1 Xenova Ltd: Microbial screen

(i) Background

Xenova Group plc is a biopharmaceutical company specialising in the discovery and development of new small molecule drugs derived originally from naturally occurring micro-organisms, such as fungi and bacteria, and from plants. The Company's broad-based, proprietary platform technologies enable it rapidly to screen and evaluate large numbers of chemical compounds produced by these organisms. Candidate compounds, or leads, are then identified for development as new drugs for the treatment of a wide range of diseases, such as cancer, cardiovascular conditions, central nervous system disorders as well as serious chronic conditions, such as immune inflammatory disorders.

(ii) Microbial Resources

Of the top-selling drugs world-wide in 1993, 24 were small molecules and, of these, 7 were derived originally from naturally occurring micro-organisms (microbes). Most microbial chemicals have inherent biological activity, making microbes a rich and inexhaustible source of drug leads for new therapeutic applications. Fungi alone have already proved to be a source of many valuable drugs, including *beta* lactam antibiotics for infectious diseases and cyclosporin for organ transplantation. Xenova has isolated approximately 25800 micro-organisms to date, and has access to over 6800 species of plants. The company's resources in fungi, bacteria and lichens, as well as higher plants, provide a vast and largely untapped supply of structurally diverse chemical compounds potentially capable of arresting a wide variety of disease processes.

(iii) Extraction and evaluation of compounds

Exotic fungi and plants are collected by ecologists and brought back to Xenova from places such as Ecquador, Brazil and Thailand. The fungi are then fermented on a

small scale in various media which are deficient in some way. For example one medium is phosphorus deficient, another nitrogen deficient and so on. The idea being that the different stress conditions will lead to the production of different secondary metabolites. These cultures are then ultra filtered through 10kDa cut-off membranes which allow the passage of liquid (broth) but leave behind the biomass, i.e. cells. Hydrophilic compounds will be present in the aqueous extract. The biomass is then extracted using 100% methanol (MeOH). This lyses the cells and allows the release of hydrophobic compounds which are also collected. The samples are dispensed into microtitre plates and stored at -20°C. The Biochemistry Department then uses these plates in screening assays. They use high-throughput technologies such as the 'TopCount' liquid scintillation counter used in the multidrug resistance screen for examining drug accumulation. Any sample which appears active will be retested and if it is consistently active it will be refermented before fractionation, using HPLC based assays, of the extract (broth or MeOH) by the Natural Product Chemistry Department. Ideally interaction between scientists in both the Biochemistry and the Natural Product Chemistry Departments by assay-guided fractionation leads to the identification of the active compound. This 'active' is then passed to the molecular pharmacologists who evaluate the compound in mechanism-based secondary assays. It is at this stage that I receive compound for evaluation in our laboratory in Cambridge. If the compound continues to show promising activity in these cell-based secondary assays it may become the subject of a medicinal chemistry programme to identify more potent 'actives' with increased solubility and an improved toxicity profile. If the compound cannot be synthetically synthesised easily it may have to be isolated from large fermentations of the original organism (1000's of litres) before semi-synthetic modification. Very promising compounds ('lead compounds'), are then evaluated by the Pharmacology Department in *in vivo* models. This lengthy extraction and evaluation procedure will hopefully result in the identification of compounds suitable for progression into preclinical research and development followed by phase I clinical trials.

4.1.2 Classification of modifiers of P-glycoprotein-mediated multidrug resistance

(i) Introduction

The following section focuses on compounds that have been identified as chemosensitisers and have potential clinical use based on their availability, prior clinical use and toxicological profile

(ii) Calcium channel blockers

Tsuruo's group was the first to show the ability of verapamil to reverse resistance in murine MDR cells (Tsuruo *et al.*, 1981). Subsequently many investigators have demonstrated the chemosensitising activity of verapamil in various rodent and human MDR cell lines (Ford and Hait, 1990). A number of groups have shown that photoactivated verapamil analogues bind irreversibly to P-glycoprotein and that verapamil inhibits the binding of many chemotherapeutic drugs as well as other chemosensitisers to P-glycoprotein (Cornwell *et al.*, 1987; Safa *et al.*, 1988), suggesting that the mechanism of action of verapamil is through blocking the binding of drugs to P-glycoprotein. In terms of clinical potential for MDR modulation, verapamil is limited by its cardiovascular effects in humans at plasma concentrations in the 2 to 6 μM range needed for antagonism of MDR *in vitro* (Candell *et al.*, 1979). In a screen of 14 structural analogues of verapamil, the compound Ro11-2933 was found to be 10-fold more potent than verapamil for sensitising MDR murine leukaema cells to doxorubicin (Kessel *et al.*, 1985). This agent is also less toxic *in vivo* to mice than verapamil. In addition the plasma levels of Ro11-2933 achieved *in vivo* (less than 2μM) were effective in vitro, this agent is a promising candidate for clinical trials in humans. It was originally thought that the activity of these compound in MDR cells may be related to the calcium channel blocking activity of the drugs. Nifedipine, however, is known to be a potent calcium channel blocker but is a poor antagonist of MDR (Ramu *et al.*, 1984). Conversely, the dihydropyridine analogue, PAK-200 possesses very low calcium channel activity, yet it fully reversed resistance

to vincristine at a dose of 5μM in a human MDR cell line (Niwa *et al.*, 1992). This indicates that there is a lack of correlation between calcium channel antagonism and anti-MDR activity. The use of less cardiotoxic enantiomers of verapamil (D-verapamil) and its analogues may also provide a means for achieving clinically effective MDR reversal in the clinic.

(iii) Calmodulin antagonists

The second class of MDR modifiers to be identified were drugs known to inhibit calmodulin-mediated processes. The phenothiazine calmodulin antagonist, trifluoperazine, caused a 10-fold increase in doxorubicin sensitivity in 40-fold resistant murine cells and a 5-fold increase in doxorubicin accumulation, but did not significantly alter drug cytotoxicity or accumulation in sensitive cell lines (Tsuruo *et al.*, 1982). The examination of structure-activity relationships for a series of 22 phenothiazine derivatives led to the identification of the thioxanthene class of calmodulin antagonist MDR modifier (Ford *et al.*, 1989). The lead compound, *trans*-flupenthixol, reversed MDR in a number of human and murine MDR cell lines and in sensitive cells transfected with the *MDR1* gene, increased doxorubicin accumulation to a greater extent than either its stereoisomer *cis*-flupenthixol or verapamil, and inhibited photoactive azidopine binding to P-glycoprotein (Ford *et al.*, 1990). *Trans*-flupenthixol has virtually no effect as a dopamine antagonist and as a result lacks *in vivo* toxicity due to extrapyramidal side effects (Johnstone *et al.*, 1978). This drug may, therefore, be uniquely suited for *in vivo* use as a MDR modifier.

(iv) Steroids and hormonal analogues

The expression of high levels of P-glycoprotein in the human adrenal cortex and placenta (Arceci *et al.*, 1988) suggests a possible role for the pump in the physiological transport of steriod hormones. Several groups have, therefore, studied whether steriods are natural substrates for P-glycoprotein or are chemosensitisers in combination with cytotoxic drugs. Progestorone increased the accumulation and

enhanced the cytotoxicity of vinblastine in murine MDR macrophages that overexpress the *mdr1b* gene (Yang *et al.*, 1989 & 1990). It also inhibited the binding of vincristine and vinblastine to membranes prepared from MDR cells (Naito *et al.*, 1989). Interestingly, unlike most other resistance modifiers, progesterone does not appear to be a substrate for P-glycoprotein (Ueda *et al.*, 1992). This indicates that the ability of a compound to act as a chemosensitiser and inhibit transport of other drugs by P-glycoprotein is not necessarily related to its own ability to function as a substrate. Megesterol acetate (Megace), an orally active congener of progesterone, functions as an MDR modifier. Paradoxically, Megace increased the binding of a photoactive calcium channel blocker to P-glycoprotein by up to 2-fold but inhibited labelled *Vinca* alkaloid binding to P-glycoprotein by 50% (Fleming *et al.*, 1992). These data strongly suggest that separate drug binding sites exist on P-glycoprotein for certain chemosensitising compounds and drug substrates.

Several groups have demonstrated that synthetic steroid analogues, such as tamoxifen and the related antioestrogen toremifene are active chemosensitisers at concentrations of 2 to 10μM in a number of P-glycoprotein overexpressing MDR cell lines (Foster *et al.*, 1988; Degregorio *et al.*, 1989). In the clinic, high serum levels of these antioestrogens can be achieved due to their lack of side effects (at least during short term use). This suggests that they may be well tolerated, effective chemosensitisers when used in combination with cytotoxic agents for the treatment of clinical drug resistance.

(v) Cyclosporins

Cyclosporin A, a hydrophobic cyclic peptide of 11 amino acids that is widely used in human organ transplantation, has been shown by several groups to reverse resistance in MDR (Slater *et al.*, 1986a & b; Twentyman *et al.*, 1987a; Hait *et al.*, 1989). It has been reported that cyclosporin A itself is accumulated less in P-glycoprotein overexpressing cells (Goldberg *et al.*, 1988). Cyclosporin A enhanced vincristine

toxicity in both sensitive and vincristine-resistant Chinese hamster MDR cells, but by a 10-fold greater factor in the MDR cells. It completely reversed the 22-fold decreased level of vinblastine accumulation displayed by the MDR cells, but it also caused a 2-fold increase in vinblastine accumulation in the sensitive cells (Tamai *et al.*, 1991). These data suggest that cyclosporin A may serve as a P-glycoprotein substrate and antagonise MDR at least in part via competitive inhibition of P-glycoprotein-mediated outward transport of cytotoxic drugs. They also imply, however, that cyclosporin A may modulate cytotoxicity via mechanisms other than competitive inhibition. Because of the profound immunosuppressive effects of cyclosporin A, there has been great interest in exploring the resistance modifying ability of other less immunosuppressive and nephrotoxic cyclosporin analogues. Researchers at Sandoz discovered that the cyclosporin D analogue, PSC 833, was 10-fold more potent than cyclosporin A. PSC 833 (1μM) has been shown to cause nearly complete reversion of drug resistance to taxol in rodent cells, and it was 30-fold more potent than verapamil (Gaveriaux *et al.*, 1991).

In summary, the newly developed nonimmunosuppressive cyclosporin analogues, particularly PSC 833, appear to be good candidates for drug resistance modifying agents in the clinic owing to their increased potency and decreased toxicity.

(vi) Miscellaneous compounds

Numerous compounds have been identified in the search for agents which circumvent MDR that do not belong to any of the previously discussed classes. Most of these agents are lipophilic in nature and they share a broad structural similarity that includes a heterocyclic ring separated at a distance from a cationic, amino group. Examples of this diverse group of resistance modifiers include: antiarrhythmics such as amiodarone (Chauffert *et al.*, 1986) and quinidine (Tsuruo *et al.*, 1984); antimalarials such as chloroquine and quinacrine (Zamora *et al.*, 1988); the indole alkaloid, reserpine (Beck *et al.*, 1988); the antihistamine, terfenadine (Hait *et al.*, 1993); and antibiotics such as erythromycin (Hofsi *et al.*, 1989), cefoperazone (Gosland *et al.*, 1989) and tetracycline (Kavallaris *et al.*, 1993).

There are two relatively new resistance modifiers that are of particular interest. Firstly, the acridone carboxamide derivative, GF120918, possesses remarkable potency for reversing MDR in several human and rodent MDR cell lines. GF120918 is active at concentrations as low as 0.02μM displaying similar activity to 5μM verapamil (Hyafil *et al.*, 1993). Secondly, a novel class of compounds active against MDR has been identified by screening natural products extracted from strains of cyanobacteria (blue-green algae). One of the isolates, tolyporphin, functions as a potent resistance modifier (Prinsep *et al.*, 1992). This agent sensitised MDR cells to daunomycin, vinblastine, VP-16 and taxol at doses of 0.1μM. It also inhibited binding of labelled vinblastine and azidopine to P-glycoprotein.

4.1.3 Identification of natural product diketopiperazines as modifiers of multidrug resistance

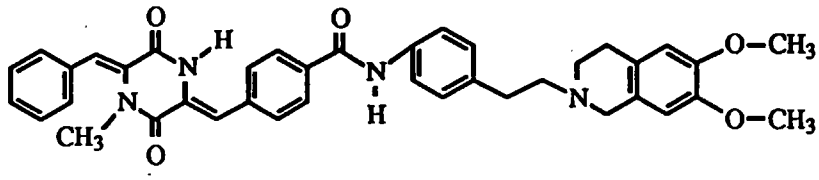
During the course of screening the Xenova microbial collection for novel modifiers of multidrug resistance scientists identified derivatives of a natural product diketopiperazine which displayed promising activity as a resistance modifier (Figure 4.1). Previously, a group in Japan isolated a substance from the organism,

Streptoverticillium aspergilloides which they discovered was a complex of two components, designated piperafizine A and B, structurally classified as the diketopiperazine family. Furthermore they demonstrated that compound A markedly enhanced the cytotoxicity of vincristine in Moser cells, a human colon carcinoma cell line with intrinsically high levels of P-glycoprotein, and moderately in the vincristine resistant P388 cell line. Piperafizine B showed a slight increase in the toxicity of vincristine only in the vincristine resistant P388 cells (Kamei *et al.*, 1990). In leukaemia-bearing mice, piperafizine A significantly increased life span when used in combination with vincristine. We now have a large number of these diketopiperazine derivatives synthesised at Xenova. After primary screening at Xenova the most promising compounds were tested in our laboratory with the objective of elucidating their mechanism of action as well as forming a more detailed profile of their activity in combination with a variety of cytotoxic drugs and in a number of cell lines. The structures of the XR compounds studied in this Chapter are shown in figure 4.1.

Figure 4.1

Structure of diketopiperazine analogues used in this study

XR9006



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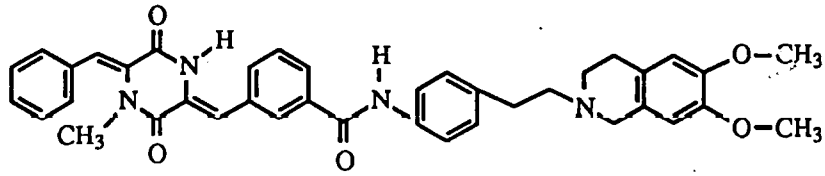

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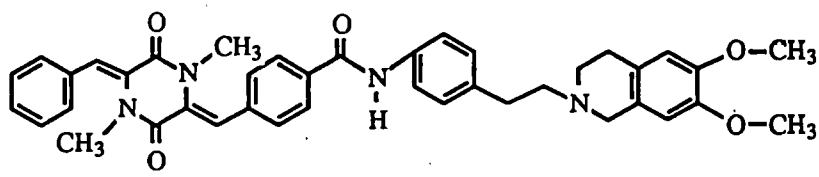

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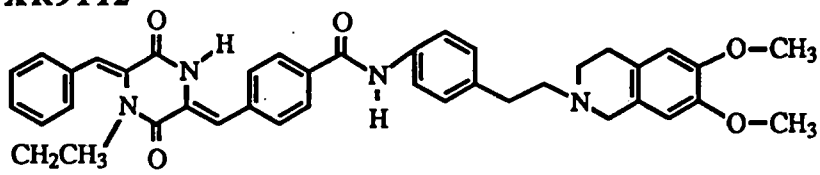
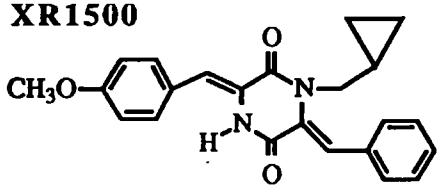
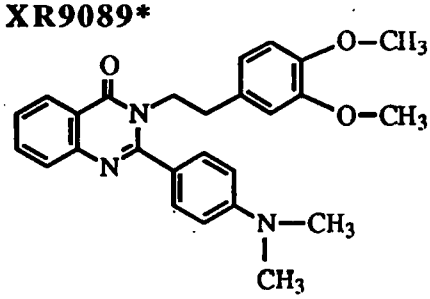


XR1500



XR9089*

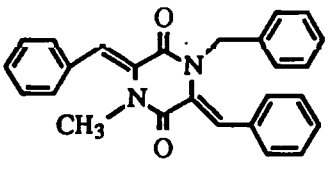


*XR9089 is not a diketopiperazine.

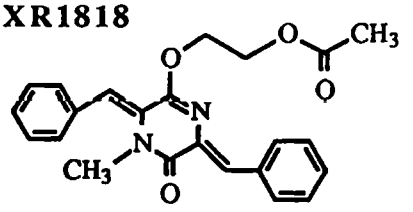
It was discovered as an impurity of a commercially supplied compound.

Figure 4.1 (continued)

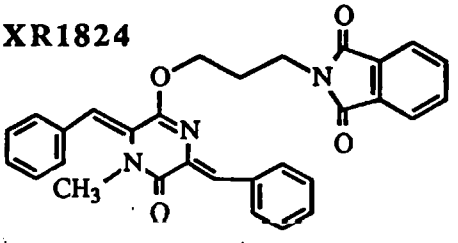
XR1779



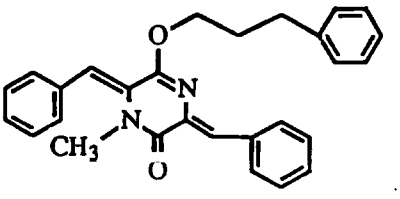
XR1818



XR1824



XR1829



4.1.3 Aims

(i) To establish the activity of a group of novel fungal product diketopiperazines as modifiers of P-glycoprotein mediated multidrug resistance.

(ii) To compare their activity in a number of different multidrug resistant cell lines and in combination with a variety of different drugs and to build a structure activity relationship.

(iii) To elucidate their mechanism of action.

4.2 Materials and Methods

4.2.1 Cell Lines

We used the human T-lymphoblastoid leukaemia cell line CEM and the human small cell lung cancer cell line H69/P together with their P-glycoprotein overexpressing sublines CEM/VLB and H69/LX4. For cell culture conditions see Section 2.1.

4.2.2 Drug Sensitivity Testing.

The cytotoxicity of the Xenova compounds were determined using the tetrazolium (MTT) reduction colorimetric assay. This method is described in detail in Section 2.3.

4.2.3 Drug Accumulation Studies

The effect of the Xenova compounds on the ability of CEM and CEM/VLB cells to accumulate [^{3}H]daunorubicin and [^{3}H]colchicine was determined as described in Section 2.5.2.ii.

4.2.4 Photoaffinity Labelling

The effect of the Xenova compounds on the ability of [^{3}H]azidopine to photolabel P-glycoprotein in membrane protein prepared from drug sensitive CEM and P-glycoprotein overexpressing CEM/VLB cells was determined as described in Section 2.6.2.

4.2.5 Confocal microscopy

The effect of the Xenova compounds (5μM) on the distribution of 10μM doxorubicin in CEM and CEM/VLB cells was determined as described in Section 2.8.2.ii.

4.3 Results

4.3.1 Toxicity of XR compounds in cell lines CEM, H69/P and L23/P together with their respective MDR cell line variants CEM/VLB, H69/LX4 and L23/R

Table 4.1 shows the toxicity of all 10 XR compounds in the parental CEM and H69/P cell lines together with their P-glycoprotein-overexpressing resistant variants CEM/VLB and H69/LX4 and in the parental cell line L23/P together with its MRP-overexpressing variant L23/R. The highest concentration of XR compound used in the studies presented in this chapter was 20μM. We received the stock solutions dissolved in DMSO at a concentration of 5mM. We applied an upper concentration limit of 20μM as many of the compounds came out of solution when dissolved in medium at concentrations higher than 20μM. We also wished to avoid having high concentrations of solvent in our experiments. The IC_{50}s for compounds that are not toxic at concentration of 20μM are, therefore, not precisely defined. Table 4.1 demonstrates that, where IC_{50}s are precisely defined in the parental and resistant cell lines, it is possible to calculate the resistance factor (resistance factor is defined by the IC_{50} of the drug in the resistant line divided by the IC_{50} of the drug in the sensitive line). The resistance factors indicate that the resistant cell lines are modestly cross resistant to the XR compounds. XR9112 was the most toxic compound in all the cell lines. The general toxicity profile for the compounds was similar in all 6 cell lines. There were, however, some exceptions: for example, CEM, CEM/VLB, L23/P and L23/R are more sensitive to XR1824 than H69/P and H69/LX4.

Table 4.1

Toxicity of XR compounds in sensitive and drug resistant cell lines

(a) CEM and CEM/VLB

	$IC_{50}(\mu M)$		
XR COMPOUND	**CEM**	**CEM/VLB**	**Resistance factor**
XR1500	>20*	>20*	N/D
XR9006	11.0 13.3	>20 14.0	N/D
XR9112	1.5 (0.7) n=3	2.9 (0.2) n=3	1.9
XR1824	5.7 (3.0) n=3	8.0 (2.9) n=3	1.4
XR1829	>20*	>20 >20 11.2	N/D
XR9051	>20*	>20*	N/D
XR9089	>20*	>20*	N/D
XR1818	7.3 (1.9) n=3	10.0 (2.0) n=3	1.4
XR1779	10.8 (0.2) n=3	12.7 (2.4) n=3	1.2
XR9019	9.1 14.0	16.3 >20	N/D

Values represent the mean (standard error) from n independent experiments. Where n<3, individual values are shown. *20μM was the highest concentration studied, the IC_{50} value was >20 in at least 3 independent experiments. IC_{50} represents the concentration of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

N/D-not determined. Resistance factor is defined by the IC_{50} of the drug in the resistant line divided by the IC_{50} of the drug in the sensitive line.

(b) H69/P and H69/LX4

	IC50(μM)		
XR COMPOUND	H69/P	H69/LX4	Resistance factor
XR1500	>20*	>20*	N/D
XR9006	>20 14.0 11.5	>20*	N/D
XR9112	2.5 (0.7) n=3	3.5 (1.5) n=3	1.4
XR1824	12.1 (3.2) n=3	14.8 (2.3) n=3	1.2
XR1829	>20*	>20*	N/D
XR9051	>20*	>20*	N/D
XR9089	>20*	>20*	N/D
XR1818	9.7 (0.7) n=3	14.5 (2.4) n=3	1.5
XR1779	7.6 (1.9) n=3	9.4 (1.0) n=3	1.2
XR9019	14.5 (1.9) n=3	>20*	>1.4

Values represent the mean (standard error) from n independent experiments. Where n<3, individual values are shown. *20μM was the highest concentration studied, the IC_{50} value was >20 in at least 3 independent experiments. IC_{50} represents the concentration of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

N/D-not determined. Resistance factor is defined by the IC_{50} of the drug in the resistant line divided by the IC_{50} of the drug in the sensitive line.

(c) L23/P and L23/R

	IC50(μM)		
XR COMPOUND	L23/P	L23/R	Resistance factor
XR1500	>20*	>20*	N/D
XR9006	>20*	>20*	N/D
XR9112	3.0 (1.2) n=3	3.5 (0.9) n=3	1.2
XR1824	4.1 (0.4) n=3	4.6 (0.8) n=3	1.1
XR1829	>20*	>20*	N/D
XR9051	>20*	>20*	N/D
XR9089	>20*	>20*	N/D
XR1818	4.6 (1.6) n=3	5.5 (1.4) n=3	1.2
XR1779	12.7 (2.0) n=3	14.3 (2.9) n=3	1.1
XR9019	10.9 (2.7) n=3	14.2 11.3 >20	N/D

Values represent the mean (standard error) from n independent experiments. Where n<3, individual values are shown. *20μM was the highest concentration studied, the IC_{50} value was >20 in at least 3 independent experiments. IC_{50} represents the concentration of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

N/D-not determined. Resistance factor is defined by the IC_{50} of the drug in the resistant line divided by the IC_{50} of the drug in the sensitive line.

4.3.2 Sensitisation of CEM, CEM/VLB, H69/P and H69/LX4 cells to doxorubicin, colchicine and taxol by the XR compounds

We examined the effect of 1 and 5μM of each of the XR compounds on the sensitivity of the parental cell lines CEM and H69/P and their respective P-glycoprotein-overexpressing sublines CEM/VLB and H69/LX4. In the case of XR9112, due to its toxic effects, we used concentrations of 0.2 and 1μM. Sensitisation ratios are shown in table 4.2 (a-e) for the compounds at 1 and 5μM (0.2 and 1μM for XR9112) in combination with colchicine, doxorubicin and taxol (taxol is only shown in CEM and CEM/VLB cells). Sensitisation ratio is defined by the ratio of IC_{50} in the presence/absence of modifier. The effect of the compounds is clearly dose and drug specific. At the higher dose of 5μM, XR9006 is the most effective modifier of colchicine resistance while XR9051 appears to be the most effective modifier of doxorubicin resistance in both CEM/VLB and H69/LX4 cells. The compounds had no effect on the sensitivity of the drug sensitive cell lines to either doxorubicin or colchicine (data not shown). At the lower concentration of 1μM, XR9006 was consistently the most effective modifier of both doxorubicin and colchicine resistance. At 1μM, XR9112 was also an effective modifier of both doxorubicin and colchicine resistance. Its toxic effects limited experimentation at higher doses and indicate that it would not be a potentially good candidate for further investigations. XR1779 and XR1818 had only modest resistance modifying activity. They are also more toxic than the more effective modifiers XR9051 and XR9006. XR1500 was one of the initial compounds to be discovered to have potential as a resistance modifier. Its effects are, however, modest in comparison to the newly synthesised analogues XR9051, XR9006 and XR9112. Initial studies with XR9019 show that it appears to be an effective modifier. Problems with its synthesis at Xenova have made further studies impossible. Interestingly, in combination with taxol, a different compound has the greatest resistance modifying activity. At a concentration of 5μM XR9089, a compound which possessed only modest resistance modifying activity in combination

with doxorubicin and colchicine, is the most effective of all the compounds in combination with taxol. This provides additional evidence that the effect of the XR compounds is dependent on the cytotoxic drug which it is used in combination with. Figures 4.2 (a-e) show graphical representations of the effect of the 2 best XR modifiers, XR9051 and XR9006 on the sensitisation of CEM/VLB to doxorubicin, colchicine and taxol.

Table 4.2

(a) Sensitisation of CEM/VLB cells to doxorubicin by XR Compounds

XR CPD	Sensitisation Ratio[1] @ 1μM XR CPD	Sensitisation Ratio[1] @ 5μM XR CPD
XR1500	1.41 (0.20) n=3	1.75** (0.03) n=3
XR9006	6.64 (2.80) n=3	21.35* (3.87) n=3
[2]XR9112	5.46 (1.69) n=3	20.44** (1.56) n=3
XR1824	1.40 (0.18) n=3	5.22* (0.91) n=3
XR1829	2.04* (0.16) n=3	3.93* (0.54) n=3
XR9051	2.99 (1.12) n=3	26.67* (4.41) n=3
XR9089	1.67 (0.67) n=3	1.25 1.40
XR1818	1.00 2.25	3.12
XR1779	1.25 2.25	1.79 4.25
XR9019	2.00	10.00

[1]Ratio of IC_{50} in presence/absence of modifier. IC_{50} represents the dose of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

*$p<0.05$ (significantly different from 1.0). **$p<0.01$ (highly significantly different from 1.0) (Student's t test). Values are means of n independent experiments.

Where n<3, individual values are shown. Parentheses show standard error.

[2]Due to toxicity of XR9112, doses of 0.2 and 0.1μM were used instead of 1 and 5μM

Table 4.2

(b) Sensitisation of H69/LX4 cells to doxorubicin by XR Compounds

XR CPD	Sensitisation Ratio[1] @ 1μM XR CPD	Sensitisation Ratio[1] @ 5μM XR CPD
XR1500	1.00 (0.00) n=3	2.83* (0.67) n=3
XR9006	14.5 (5.56) n=3	45.00* (10.41) n=3
[2]XR9112	7.08* (1.39) n=3	26.00* (3.88) n=3
XR1824	1.33 (0.33) n=3	7.42* (1.29) n=3
XR1829	1.83 (0.35) n=3	2.63 (0.57) n=3
XR9051	3.30 (1.90) n=3	57.50** (3.01) n=3
XR9089	1.67 (0.42) n=3	2.27* (0.39) n=3
XR1818	0.93 (0.07) n=3	1.10 (0.10) n=3
XR1779	1.17 (0.19) n=3	2.17 (0.49) n=3
XR9019	5.0 2.5	11.53* (1.75) n=3

[1]Ratio of IC_{50} in presence/absence of modifier. IC_{50} represents the dose of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

*$p<0.05$ (significantly different from 1.0). **$p<0.01$ (highly significantly different from 1.0) (Student's t test). Values are means of n independent experiments.

Where n<3, individual values are shown. Parentheses show standard error.

[2]Due to toxicity of XR9112, doses of 0.2 and 0.1μM were used instead of 1 and 5μM

Table 4.2

(c) Sensitisation of CEM/VLB cells to colchicine by XR Compounds

XR CPD	Sensitisation Ratio[1] @ 1μM XR CPD	Sensitisation Ratio[1] @ 5μM XR CPD
XR1500	0.62* (0.08) n=4	1.42 (0.21) n=4
XR9006	7.57* (1.27) n=3	18.38** (0.99) n=4
[2]XR9112	4.28* (1.29) n=4	14.11** (2.01) n=4
XR1824	3.46 (1.24) n=4	10.26* (3.08) n=3
XR1829	1.32 (0.24) n=4	2.84 (1.45) n=3
XR9051	1.37 (0.19) n=3	17.75** (2.88) n=4
XR9089	1.69 (0.13) n=4	4.17 (0.44) n=3
XR1818	0.72* (0.10) n=4	2.06 (1.01) n=3
XR1779	0.77** (0.03) n=4	1.52 (0.25) n=4
XR9019	4.00 1.17	10.00

[1]Ratio of IC_{50} in presence/absence of modifier. IC_{50} represents the dose of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

*$p<0.05$ (significantly different from 1.0). **$p<0.01$ (highly significantly different from 1.0) (Student's t test). Values are means of n independent experiments.

Where n<3, individual values are shown. Parentheses show standard error.

[2]Due to toxicity of XR9112, doses of 0.2 and 0.1μM were used instead of 1 and 5μM

Table 4.2

(d) Sensitisation of H69/LX4 cells to colchicine by XR compounds

XR CPD	Sensitisation Ratio[1] @ 1μM XR CPD	Sensitisation Ratio[1] @ 5μM XR CPD
XR1500	0.75* (0.061) n=4	3.25 (0.97) n=4
XR9006	8.09* (2.04) n=4	58.12* (14.12) n=4
[2]XR9112	3.10* (0.63) n=4	30.94* (10.20) n=4
XR1824	2.70 (0.80) n=4	18.75* (5.73) n=3
XR1829	1.20 (0.20) n=4	2.50 (0.74) n=4
XR9051	2.35 (0.71) n=3	42.50* (6.00) n=4
XR9089	1.89 (0.30) n=3	15.00 6.00
XR1818	0.94 (0.06) n=4	1.78* (0.33) n=4
XR1779	1.06 (0.20) n=4	3.25* (0.50) n=3
XR9019	3.42 (1.02) n=3	27.50* (6.61) n=3

[1]Ratio of IC_{50} in presence/absence of modifier. IC_{50} represents the dose of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

*$p<0.05$ (significantly different from 1.0). **$p<0.01$ (highly significantly different from 1.0) (Student's t test). Values are means of n independent experiments.

Where $n<3$, individual values are shown. Parentheses show standard error.

[2]Due to toxicity of XR9112, doses of 0.2 and 0.1μM were used instead of 1 and 5μM

Table 4.2

(e) Sensitisation of CEM/VLB cells to taxol by XR compounds

XR CPD	Sensitisation Ratio[1] @ 1μM XR CPD	Sensitisation Ratio[1] @ 5μM XR CPD
XR1500	1.60 (0.30) n=3	6.00* (0.60) n=3
XR9006	12.77* (1.52) n=3	21.70** (1.73) n=3
[2]XR9112	11.10* (2.65) n=3	17.80** (1.57) n=3
XR1824	2.00 3.50	8.00 9.10
XR1829	2.00 1.20	5.00 4.70
XR9051	2.20 (1.14) n=3	22.30** (1.30) n=3
XR9089	9.17* (2.14) n=3	26.58* (5.88) n=3
XR1818	4.17* (0.43) n=3	8.05 8.58
XR1779	1.87 (0.19) n=3	8.30 10.00

[1]Ratio of IC_{50} in presence/absence of modifier. IC_{50} represents the dose of drug required to reduce fractional absorbance to 50% of control values in the tetrazolium reduction (MTT) assay.

*$p<0.10$ (significantly different from 1.0). **$p<0.01$ (highly significantly different from 1.0) (Student's t test). Values are means of n independent experiments.

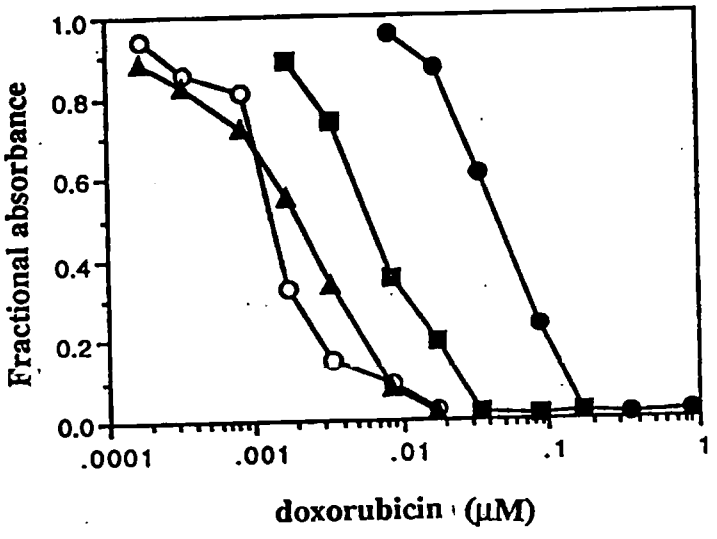
Where n<3, individual values are shown. Parentheses show standard error.

[2]Due to toxicity of XR9112, doses of 0.2 and 0.1μM were used instead of 1 and 5μM

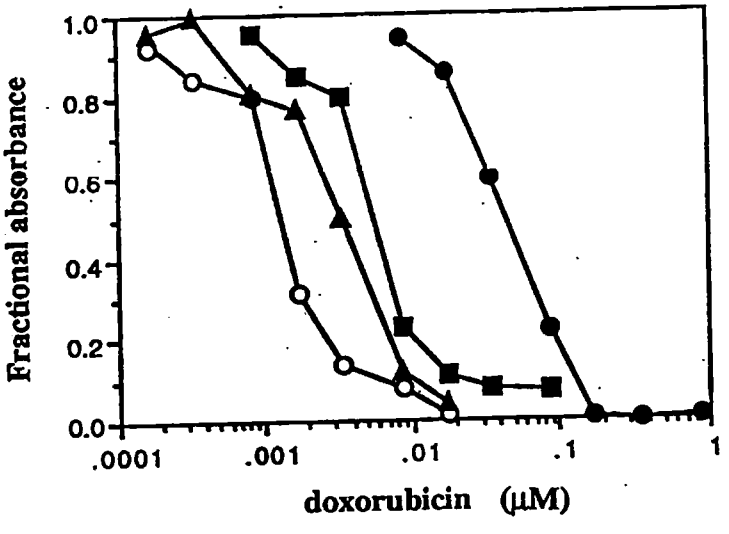
Figure 4.2

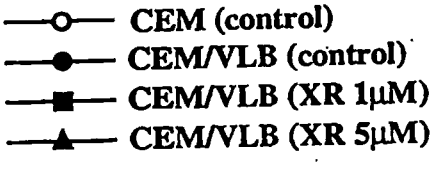
Effect of (a) XR9006 and (b) XR9051 on the sensitivity of CEM/VLB cells to doxorubicin

(a)



(b)



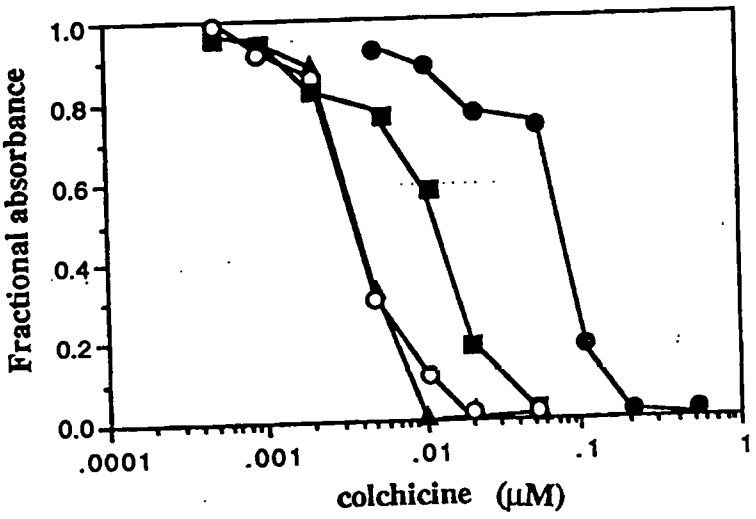


Figures represent typical data sets. Similar results were obtained in at least 3 independent experiments. Mean (standard error) for sensitisation ratios are shown in table 4.2.

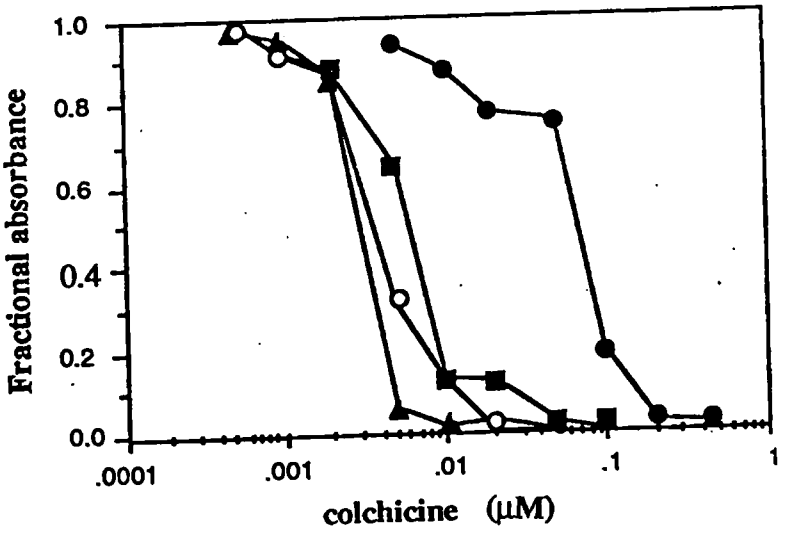
Figure 4.3

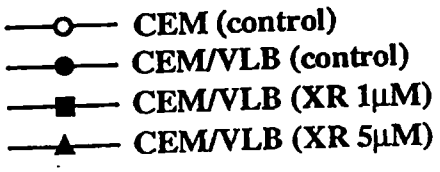
Effect of (a) XR9006 and (b) XR9051on the sensitivity of CEM/VLB cells to colchicine

(a)



(b)



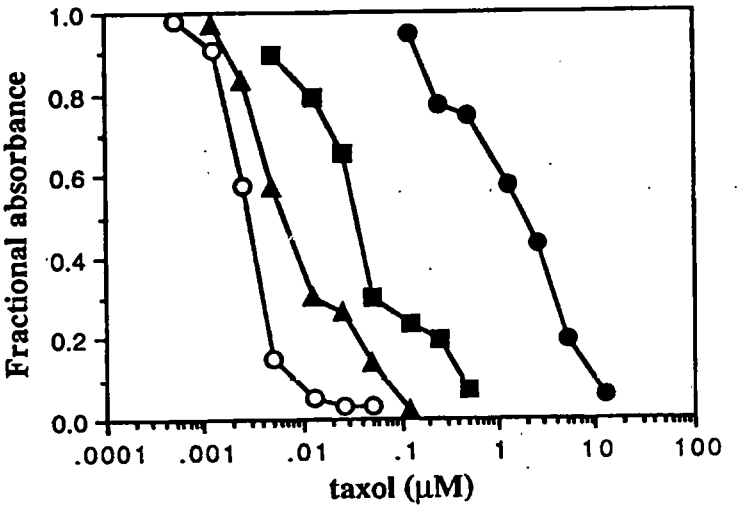


Figures represent typical data sets. Similar results were obtained in at least 3 independent experiments. Mean (standard error) for sensitisation ratios are shown in table 4.2.

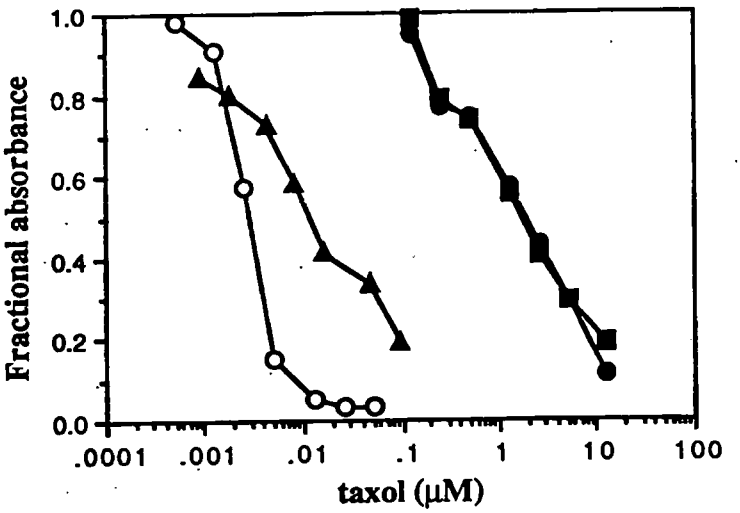
Figure 4.4

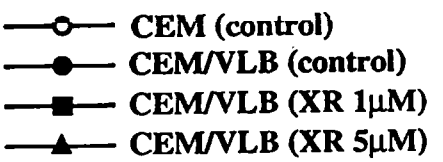
Effect of (a) XR9006 and (b) XR9051 on the sensitivity of CEM/VLB cells to taxol

(a)



(b)





Figures represent typical data sets. Similar results were obtained in at least 3 independent experiments. Mean (standard error) for sensitisation ratios are shown in table 4.2.

4.3.3. Effect of XR compounds on the accumulation of [^{3}H]daunorubicin and [^{3}H]colchicine in CEM and CEM/VLB cells

The results of drug accumulation studies confirm those of the sensitisation experiments. The effects of the XR compounds on accumulation of tritiated drug appears to be dependent on the drug used. XR9006 at concentrations of 1 and 10μM has the greatest effect on the accumulation of [^{3}H]colchicine. In contrast XR9051 has the greatest effect on the accumulation of [^{3}H]daunorubicin. At lower concentrations of 0.1 and 1μM, however, XR9006 and XR9112 have a greater effect than XR9051 on the accumulation of [^{3}H]daunorubicin. It should be noted that the IC_{50} of XR9112 is 2.9μM in CEM/VLB cells (4 day exposure) and therefore the effects seen here on the accumulation of [^{3}H]daunorubicin and [^{3}H]colchicine may be due to toxic effects of the drug such as alterations in membrane permeability

Table 4.3

(a) Effect of XR compounds on the accumulation of [^{3}H]colchicine in CEM/VLB cells.

	ACCUMULATION RATIO[1]		
XR CPD	XR 0.1μM	XR 1.0μM	XR 10.0μM
XR1500	1.22* (0.05) n=4	1.08 (0.09) n=3	1.33 (0.27) n=3
XR9006	1.46** (0.05) n=4	2.51* (0.46) n=3	4.94* (0.66) n=3
XR9112	1.68* (0.20) n=4	3.98* (0.92) n=3	4.51** (0.24) n=3
XR1824	1.29 (0.24) n=3	1.00 (0.12) n=3	3.04 (0.88) n=3
XR1829	1.07 (0.14) n=3	0.94 (0.30) n=3	1.68 (0.33) n=3
XR9051	1.11 (0.15) n=3	0.89 (0.11) n=3	4.18* (1.20) n=3
XR9089	1.11 (0.12) n=3	1.37 (0.26) n=3	1.65* (0.15) n=3
XR1818	1.37 (0.31) n=3	0.71 (0.13) n=3	1.85* (0.09) n=3
XR1779	1.32 (0.28) n=3	0.92 (0.10) n=3	1.55 (0.20) n=3

*p<0.05 (significantly different from 1.0) **p<0.01 (highly significantly different from 1.0)

(Student's t test). Values are means of n independent experiments. Parentheses show standard error.

[1]Accumulation ratio = $\frac{[^3\text{H}]\text{COL accumulation + XR}}{[^3\text{H}]\text{COL accumulation - XR}}$

Table 4.3

(b) Effect of XR compounds on the accumulation of [^{3}H]daunorubicin in CEM/VLB cells

	ACCUMULATION RATIO[1]		
XR CPD	**XR 0.1μM**	**XR 1.0μM**	**XR 10.0μM**
XR1500	1.02 (0.08) n=3	0.83 (0.10) n=3	3.00* (0.42) n=3
XR9006	2.38 (0.45) n=4	10.84* (2.00) n=4	11.89* (2.21) n=4
XR9112	5.70* (1.14) n=4	11.52* (2.48) n=4	12.04* (2.47) n=3
XR1824	1.10 (0.03) n=3	2.65 (0.77) n=4	8.88* (2.00) n=4
XR1829	1.11 (0.06) n=3	1.52 (0.09) n=3	5.78* (1.25) n=4
XR9051	1.87 (0.48) n=5	7.57 (2.66) n=5	12.17** (1.12) n=4
XR9089	1.02 (0.04) n=3	1.54 (0.23) n=3	3.25* (0.26) n=3
XR1818	0.68* (0.08) n=3	0.66* (0.10) n=3	1.80* (0.06) n=3
XR1779	0.77* (0.03) n=3	0.75* (0.06) n=3	2.78* (0.21) n=3
XR9019	1.08 (0.36) n=3	5.00** (0.06) n=3	11.12** (0.66) n=3

*p<0.05 (significantly different from 1.0) **p<0.01 (highly significantly different from 1.0)

(Student's t test).Values are means of n independent experiments. Parentheses show standard error.

[1]Accumulation ratio = $\frac{[^3H]DNR \text{ accumulation + XR}}{[^3H]DNR \text{ accumulation - XR}}$

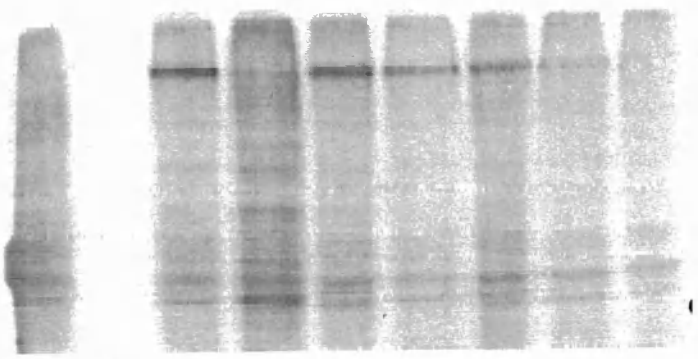
4.3.4 Effect of XR compounds on the photoaffinity labelling of P-glycoprotein in membranes

We examined the effect of the XR compounds on the ability of [^{3}H]azidopine to photoaffinity label P-glycoprotein in membranes prepared from CEM and CEM/VLB cells. We found that all the compounds inhibited [^{3}H]azidopine labelling of P-glycoprotein (at least partially) at 5 and 10μM. The more active compounds XR9051 and XR9006 appear also to inhibit partially [^{3}H]azidopine binding at the lower dose of 2μM. Figure 4.5 shows the effect of XR9051, XR9006, XR9089, XR9112 and XR1818 on the ability of [^{3}H]azidopine to photoaffinity label P-glycoprotein. XR1818 is not an active modifier but is still able to inhibit [^{3}H]azidopine labelling of P-glycoprotein at higher concentrations of 5 and 10μM. In contrast XR9051, XR9006, XR9089 and XR9112 are all active modifiers at lower concentrations (1μM) but they do not appear to inhibit [^{3}H]azidopine labelling of P-glycoprotein at this dose.

Figure 4.5

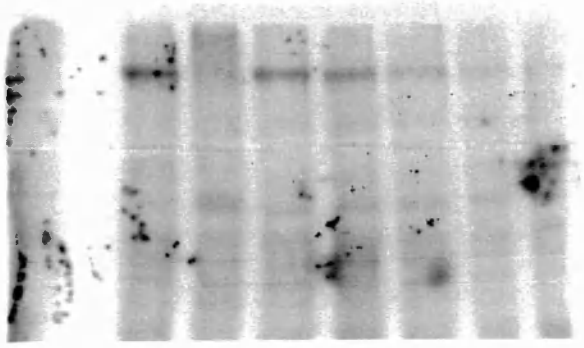
Effect of XR9006, XR9051, XR9089, XR1500 and XR1818 on the ability of [3H]azidopine to photo-label P-glycoprotein

(a) XR9006



Lane number: 1 2 3 4 5 6 7 8 9

(b) XR9051



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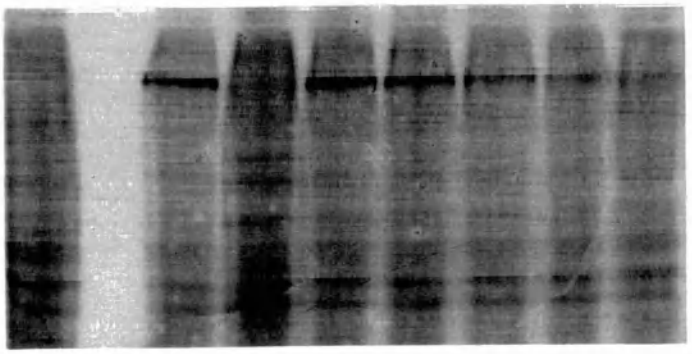
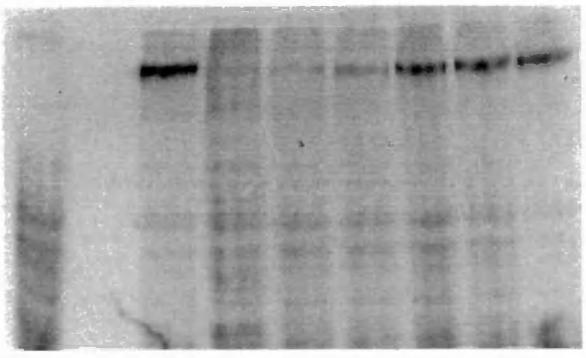
(c) XR1500



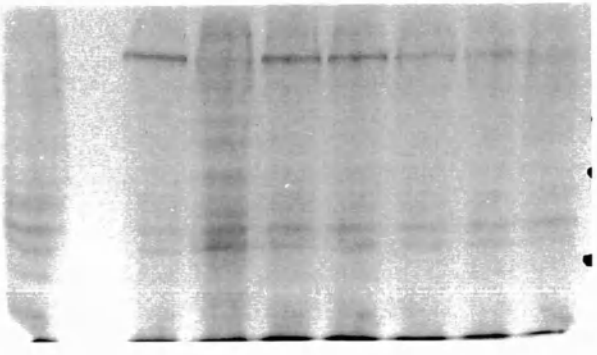
Lane number: 1 2 3 4 5 6 7 8 9

(d) XR9089



Lane number: 1 2 3 4 5 6 7 8 9

(e) XR1818



Lane number: 1 2 3 4 5 6 7 8 9

Legend:

Lane 1; CEM (parent),

Lane 2; CEM/VLB (resistant) no UV light,

Lane 3; CEM/VLB no modifier,

Lane 4; CEM/VLB + cyclosporin A 5μM,

Lane 5-9; CEM/VLB + XR compound 0.5, 1.0, 2.0, 5.0 and 10.0μM respectively

(Note for XR1818, lanes 5-9;CEM/VLB+XR compound 10.0, 5.0, 2.0, 1.0 and 0.5μM respectively)

4.3.5 Alterations in doxorubicin distribution in sensitive and multidrug resistant cells brought about by XR compounds

The photographs shown in figures 4.6 and 4.7 demonstrate the effect of XR compounds XR9051, XR9006, XR1500 and XR1818 on the distribution of doxorubicin in the drug sensitive CEM and the P-glycoprotein-overexpressing drug resistant CEM/VLB cells. Figures 4.6.a and 4.7.a show doxorubicin distribution in the parent and drug resistant cells respectively. In the drug sensitive CEM cells (Figure 4.6.a), doxorubicin appears to be mainly localised in the nucleus and the nuclear-cytoplasmic boundary. In contrast in the drug resistant CEM/VLB cells (Figure 4.7.a), doxorubicin fluorescence is reduced and there is no specific area of drug localisation within the cell. Figure 4.6.b-e show that the 4 XR compounds used here do not alter the doxorubicin distribution in the CEM cells. In the drug resistant cell line, however, all 4 of the XR compounds restore (at least partially) the pattern of doxorubicin distribution to that observed in the drug sensitive cell line (Figure 4.7.b-e). This effect is clearly dependent on the XR compound used. The more potent resistance modifiers XR9051 and XR9006 restore intracellular fluorescence in the drug resistant CEM/VLB cell line to the levels observed in the parental cell line (Figure 4.7.b & c). The same pattern of distibution is also seen, that is doxorubicin appears to be mainly localised in the nucleus and the nuclear-cytoplasmic boundary. XR1500 and XR1818 are less potent modifiers of P-glycoprotein-mediated MDR than XR9051 and XR9006. They appear to only partially restore intracellular fluorescence in the drug resistant CEM/VLB cell line (Figure 4.7.d-e). The following confocal settings were used to obtain the data presented in this chapter: 60X objective; Confocal aperture 5/15; Gain 900; Zoom 1.0; Neutral density 2, Kalman 10.

Figure 4.6.a

Subcellular distribution of doxorubicin in the drug sensitive cell line CEM

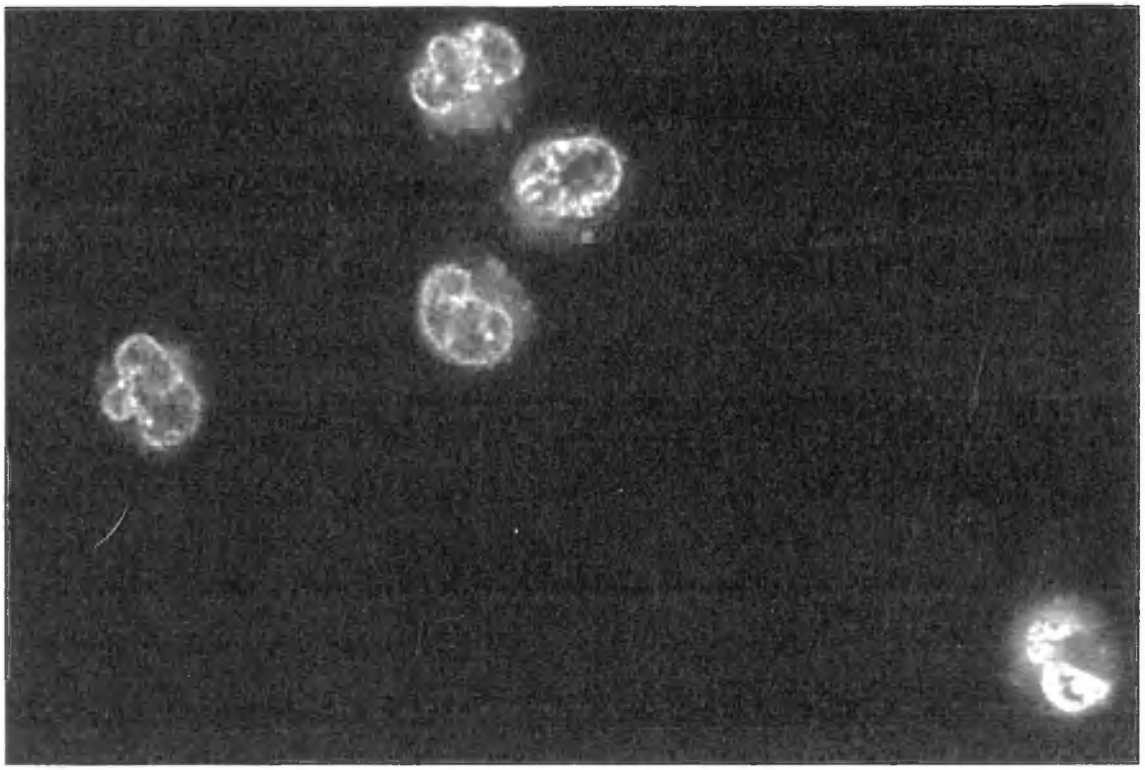
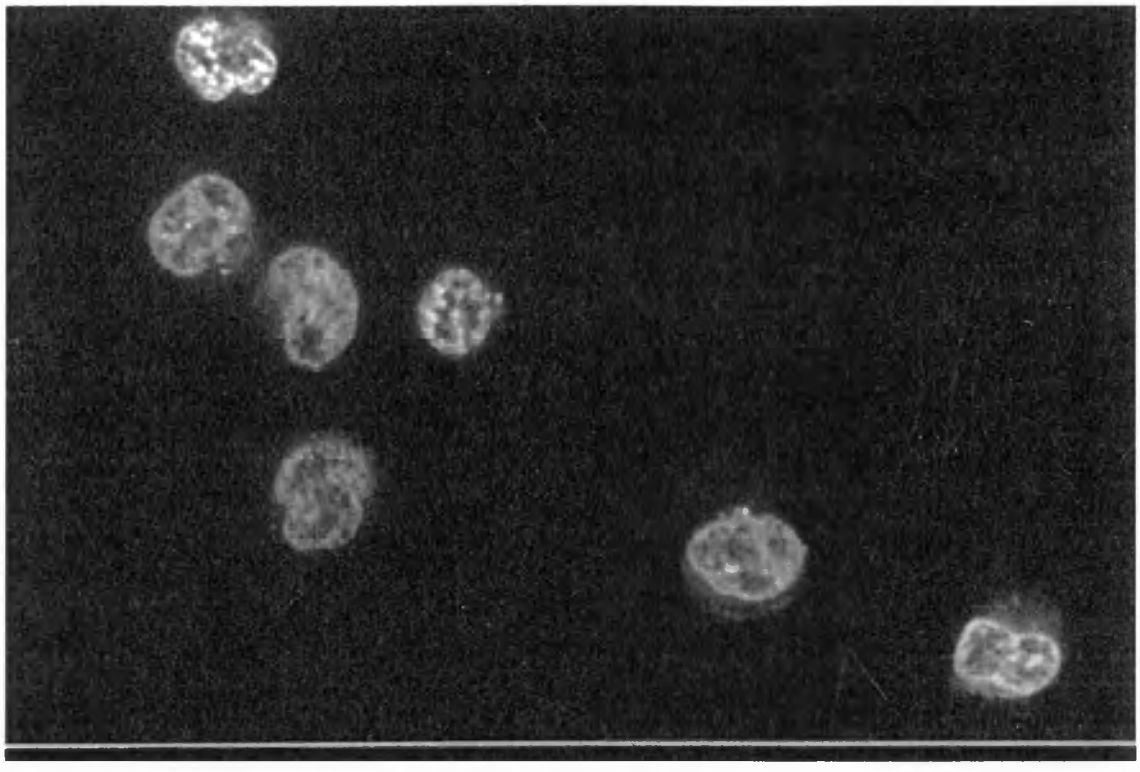


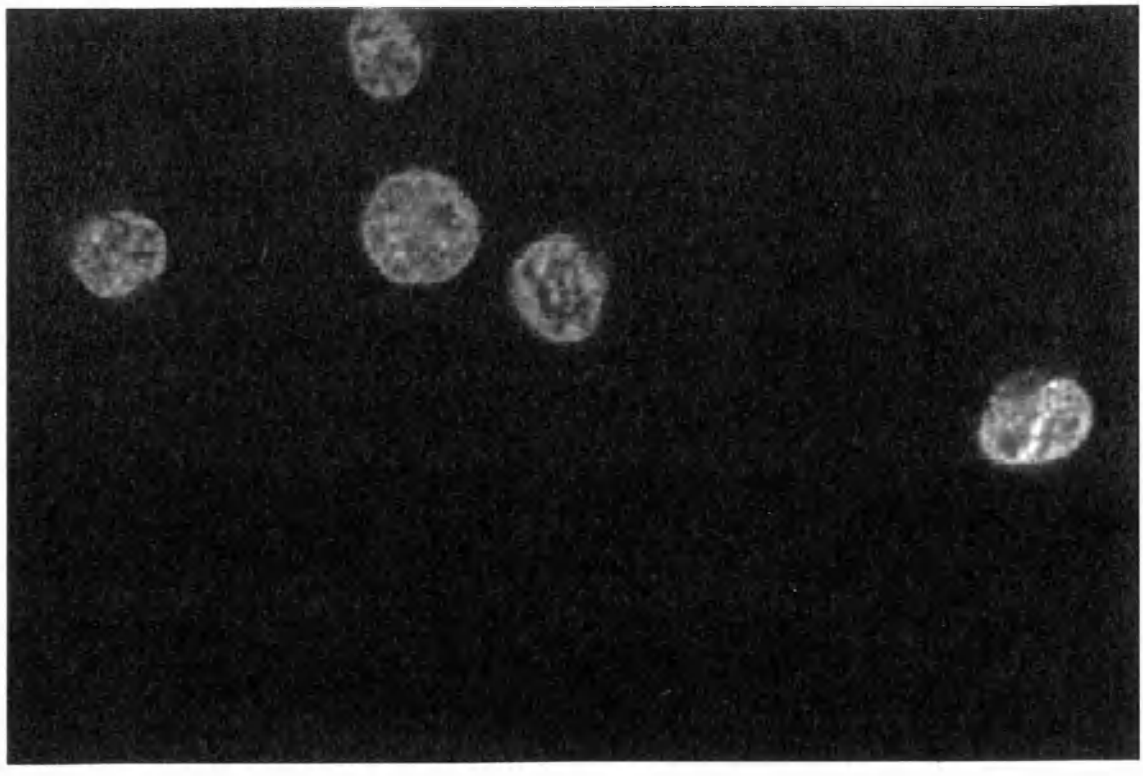
Figure 4.6.b-e

The effect of XR9051(b), XR9006(c), XR1500(d) and XR1818(e) on the subcellular distribution of doxorubicin in the drug sensitive cell line CEM

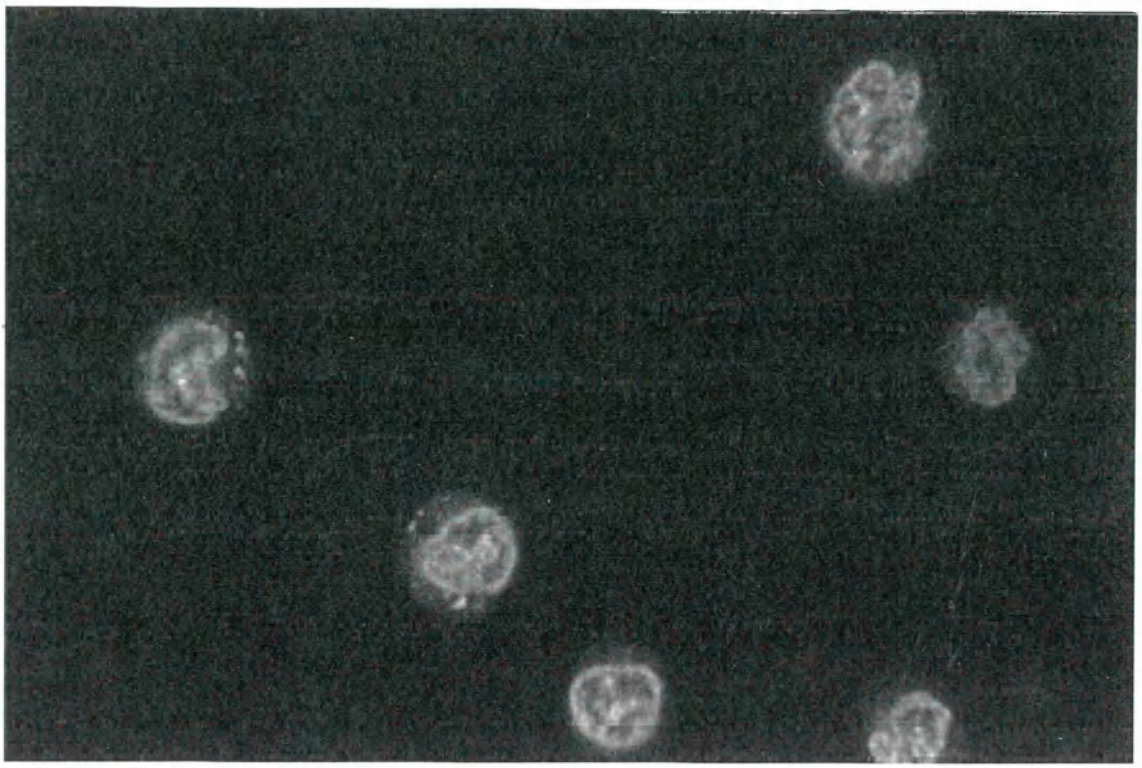
(b) XR9051



(c) XR9006



(d) XR1500



(e) XR1818

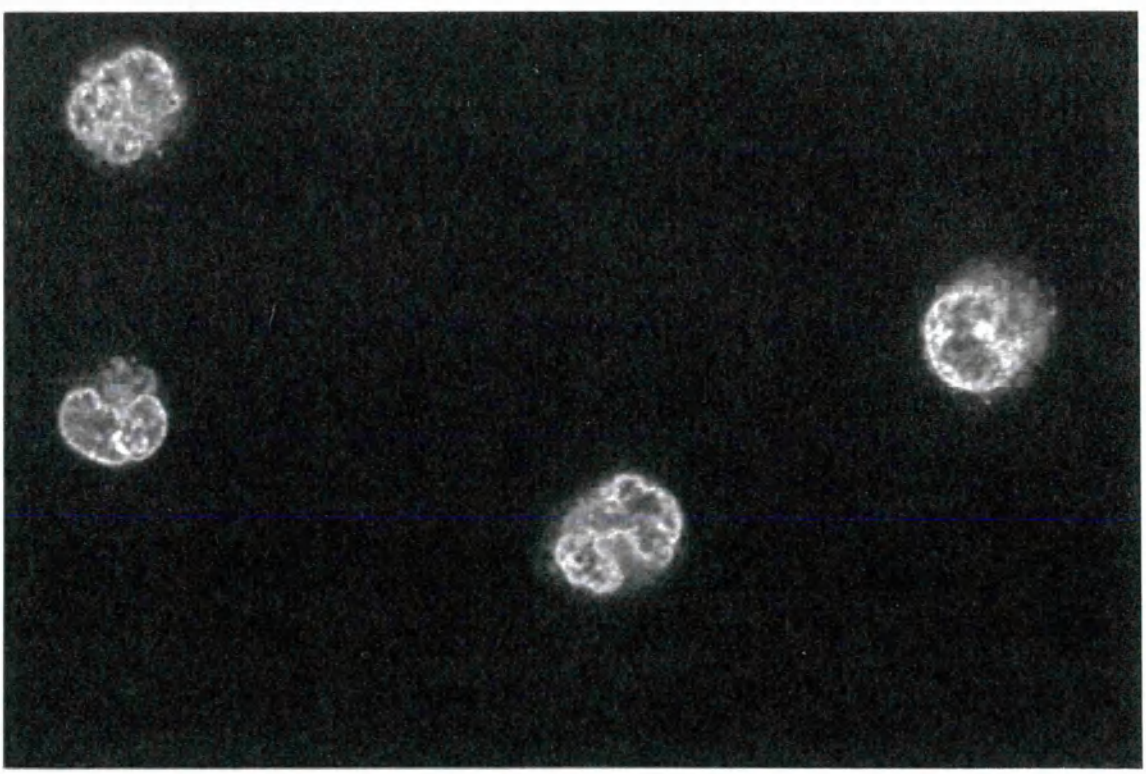


Figure 4.7.a

Subcellular distribution of doxorubicin in the drug resistant cell line CEM/VLB

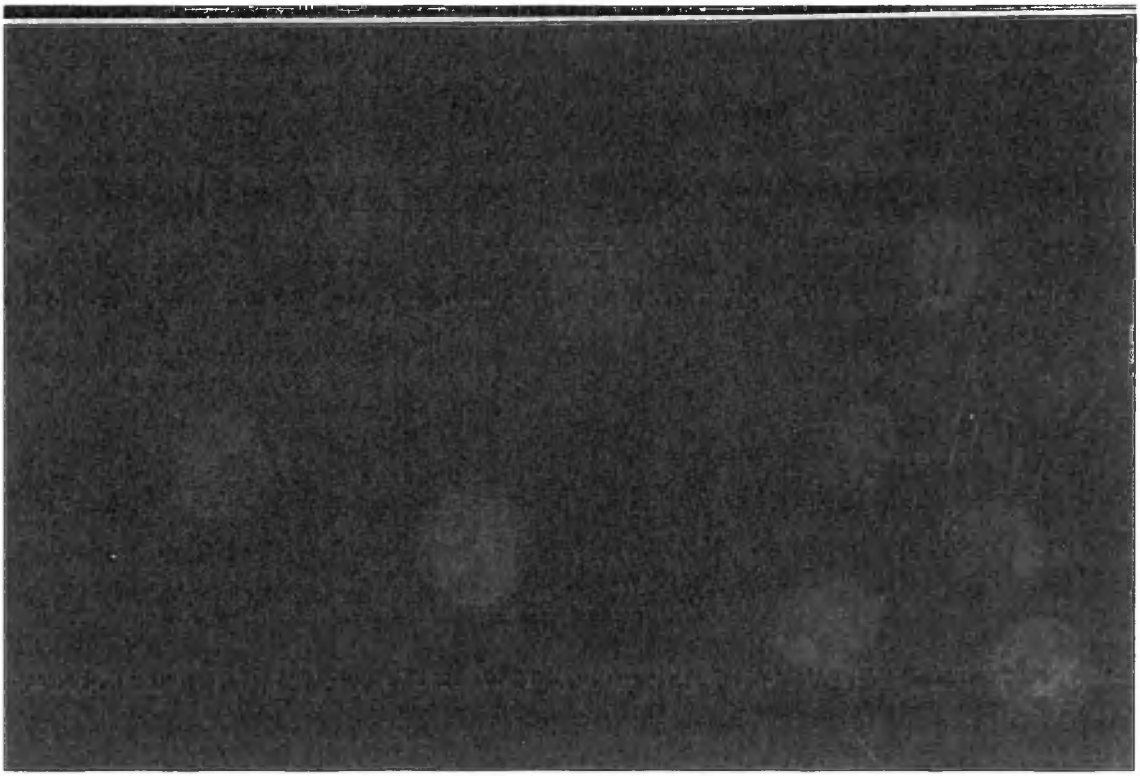
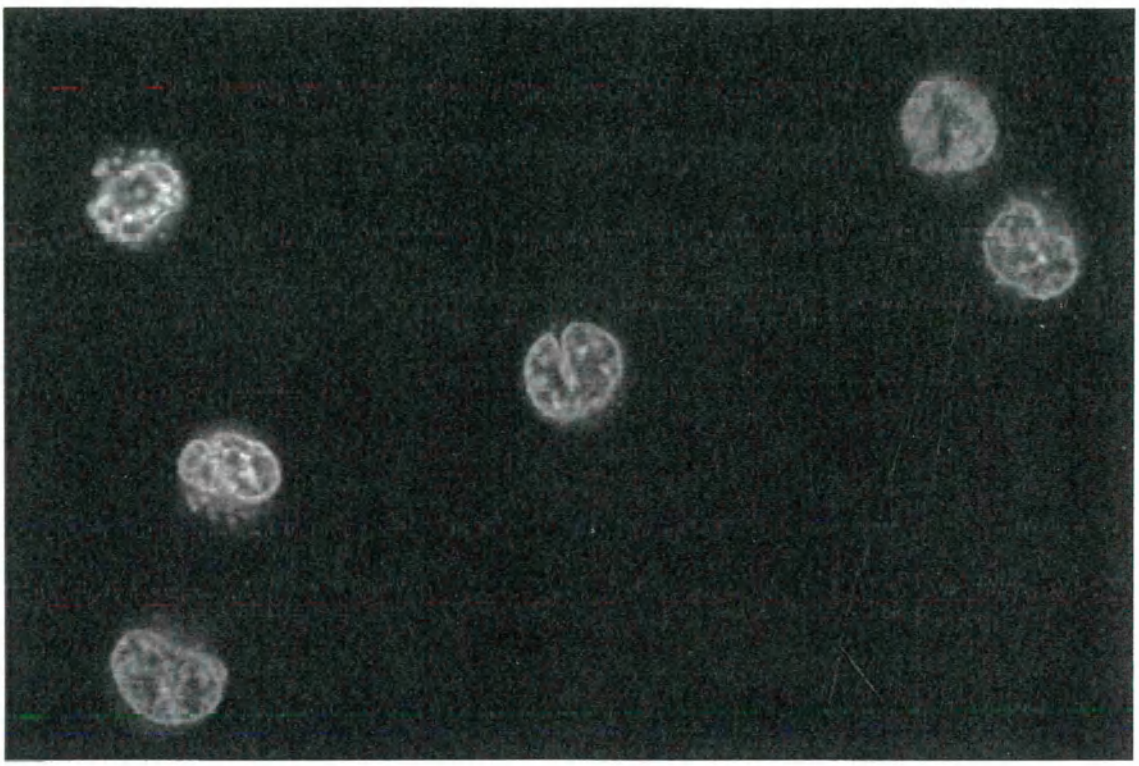


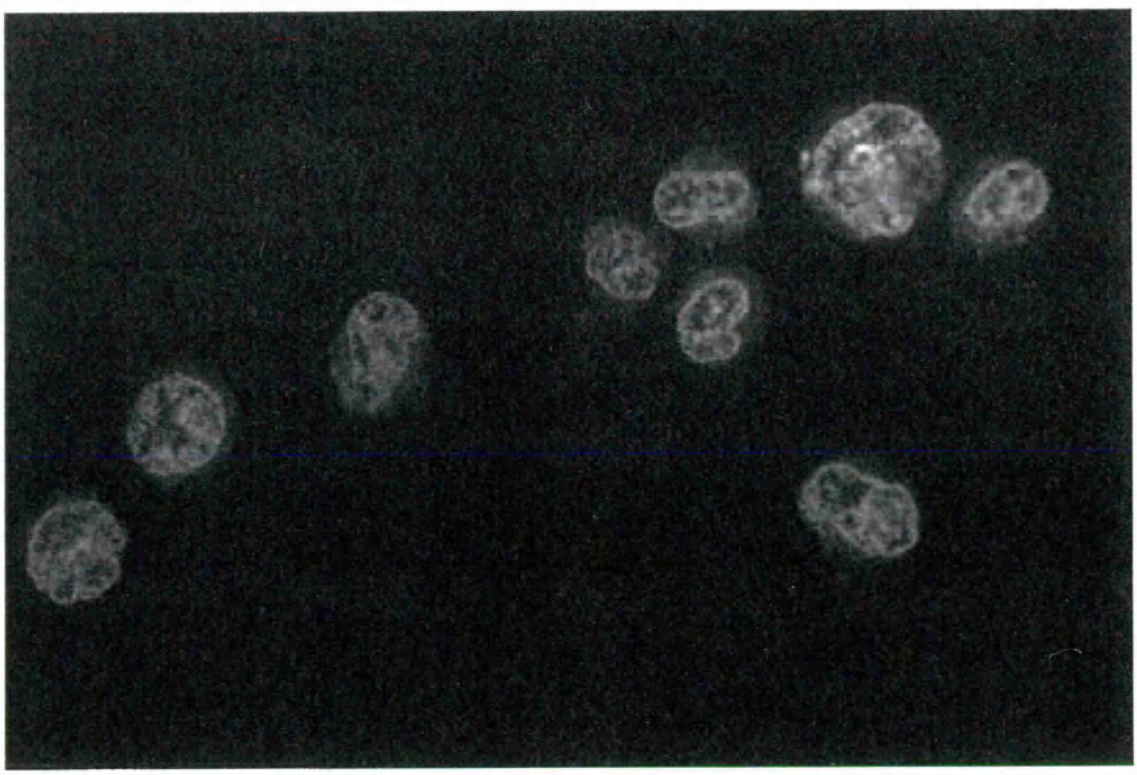
Figure 4.7.b-e

The effect of XR9051(b), XR9006(c), XR1500(d) and XR1818(e) on the subcellular distribution of doxorubicin in the drug resistant cell line CEM/VLB

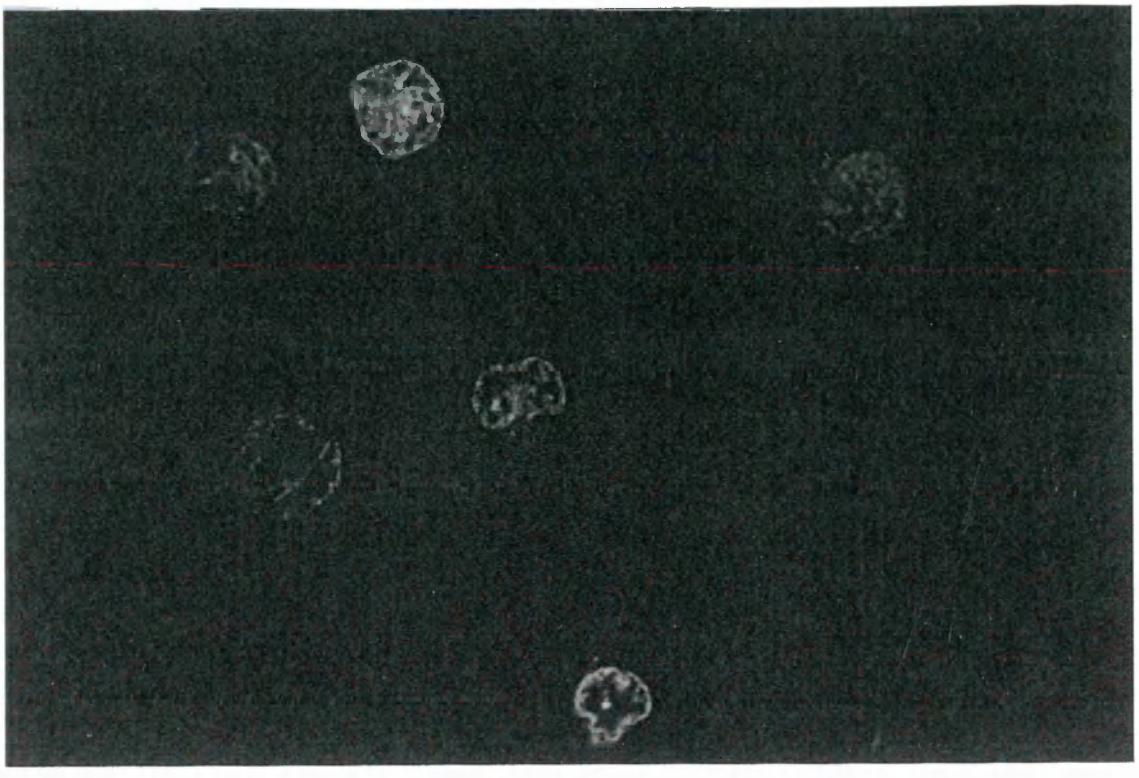
(b) XR9051



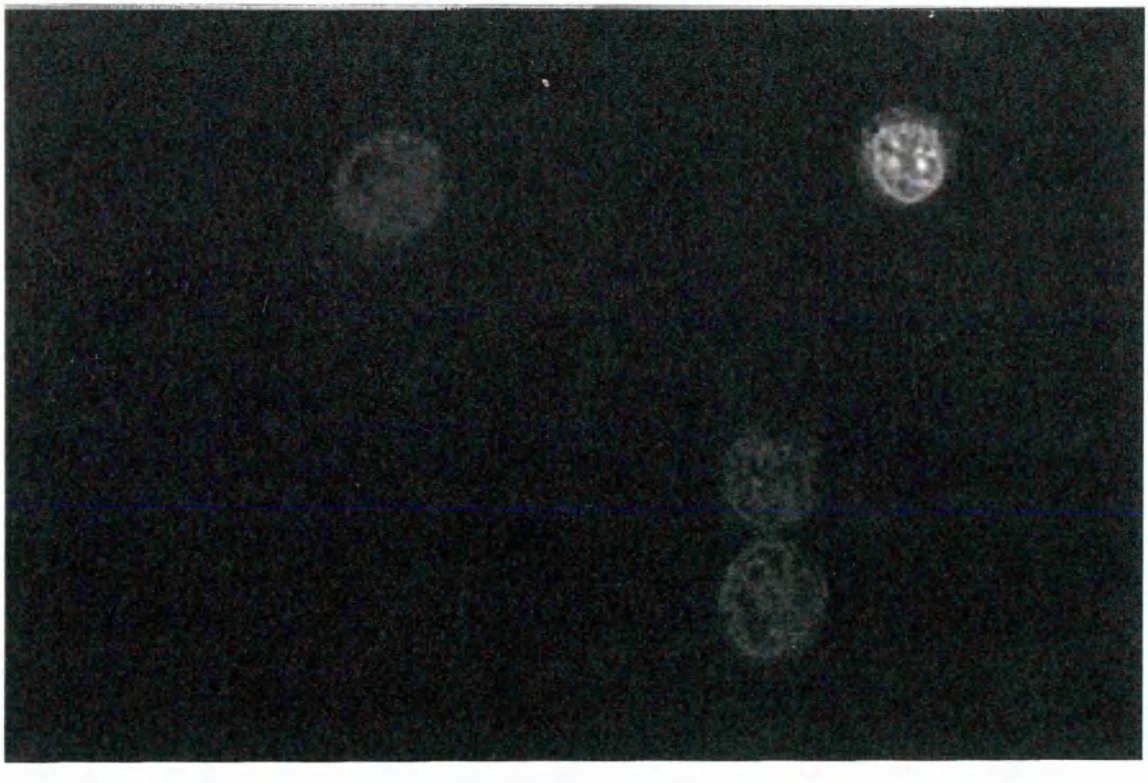
(c) XR9006



(d) XR1500



(e) XR1818



4.4 Discussion

4.4.1 General discussion

Since the original discovery of Tsuruo *et al.* (1981) that verapamil, a calcium channel blocker, was able to reverse MDR *in vitro*, a large number of compounds have been tested on MDR cell lines. It can be estimated that about 1% of organic chemicals might have this property (Robert *et al.*, 1994). Among the drug families sharing this property of reversing MDR *in vitro* are other calcium channel blockers (nicardipine, diltiazem), calmodulin inhibitors (trifluoperazine), cyclosporins, quinolines (quinine, quinidine) and numerous others (Section 4.1.3). These compounds share a high lipophilicity (generally due to the presence of several aromatic rings) and a positive charge at neutral pH (Zamora *et al.*, 1988). The development of several photoaffinity analogues of modulators or of anticancer drugs, undertaken in particular by Safa (1992), has proven that a number of modulators interact with P-glycoprotein by inhibiting its photoaffinity labelling. In our study we examined the effect of the XR compounds on the ability of [^{3}H]azidopine to photoaffinity label P-glycoprotein. We found that all the compounds inhibited [^{3}H]azidopine labelling of P-glycoprotein (at least partially) at 5 and 10μM. The more active compounds XR9051 and XR9006 appear also to inhibit partially [^{3}H]azidopine binding at the lower concentration of 2μM. Interestingly, although these modifiers are active at concentration below 2μM, they do not appear to have any effect on the ability of [^{3}H]azidopine to photoaffinity label P-glycoprotein at these low concentrations. This may be interpreted in a number of ways. Firstly, it is possible that the modifiers reverse drug resistance via a mechanism other than competitive inhibition of P-glycoprotein. They may for example, exert their action on the regulation of P-glycoprotein activity by phosphorylation: the fact that several modifiers are known inhibitors of protein kinase C was recognised long ago (Hamada *et al.*, 1987; Chambers *et al.*, 1990a & b). This seems unlikely as scientists at Xenova have found that the XR compounds do not affect PKC activity (see Chapter 7). Alternatively the compounds may be involved in drug redistribution within the cell. Secondly, the extent to which a

compound inhibits the photoaffinity labelling of P-glycoprotein by [^{3}H]azidopine may not necessarily be directly related to the reversing properties of the compound. It is likely that there is more than one drug binding site on P-glycoprotein and therefore the ability of a compound to inhibit the binding of one photoaffinity ligand does not mean that it will inhibit the binding of other photoaffinity ligands or drugs to the same extent. None of the binding sites on P-glycoprotein for anticancer drugs or modulators have been identified to date, although some domains of the protein (transmembrane domains 6 and 12) seem to be especially involved in drug binding (Greenberger *et al.*, 1993). There is even some experimental evidence against the direct interaction of some modifiers with P-glycoprotein (Jaffrezou *et al.*, 1991).

Some of the modifiers inhibited [^{3}H]azidopine photolabelling although they are not active as modifiers of MDR. This may be due to the fact that they are highly lipophilic and alter the integrity of the membrane thus preventing photolabelling.

We have studied the effect of the XR compounds on the intracellular distribution of doxorubicin by exploiting the natural fluorescence of the anthracyclines to visualise the drug using a laser scanning confocal microscope. Our results presented here indicate that there is a reduced intracellular accumulation of doxorubicin in the drug resistant CEM/VLB cells and that the XR compounds restore intracellular drug accumulation rather than altering intracellular drug distribution. This supports the theory that drug resistance in this cell line is associated with a decrease in intracellular cytotoxic drug accumulation and that the XR compounds inhibit the mechanism, probably P-glycoprotein, which causes this decreased drug accumulation.

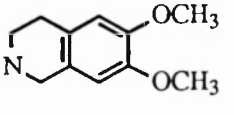
4.4.2 Structure-activity relationships

Compounds XR9006, XR9051, XR9019 and XR9112 all contain a tetrahydroisoquinoline (Figure 4.8) within their structures. This makes the molecules protonated at physiological pH (pH 7-9). Molecules that have been shown previously to be effective modifiers tend to be cationic (positively charged) lipophilic molecules (see structure of verapamil, Figure 4.9). This may explain why these compounds are the most effective modifiers of resistance in these cell lines. In contrast XR1500 contains no charge at physiological pH. Although the molecule is lipophilic it is not cationic. This explains the difference in the activity of this compound and the tetrahydroisoquinoline substituted molecules. All the molecules with the exception of XR9089 contain a diketopiperazine (Figure 4.8) within their structures. Compounds containing this structure have previously been shown to be active resistance modifiers (Kamei *et al.*, 1990).

Figure 4.8

The structure of tetrahydroisoquinoline and diketopiperazine groups

tetrahydroisoquinoline



diketopiperazine

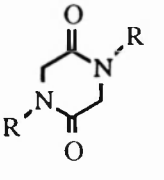
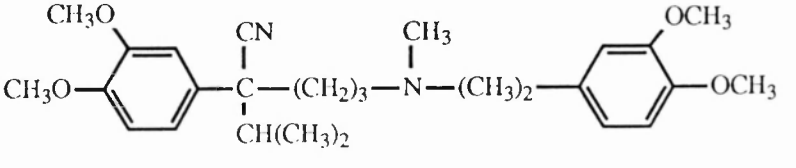


Figure 4.9

The structure of verapamil

verapamil



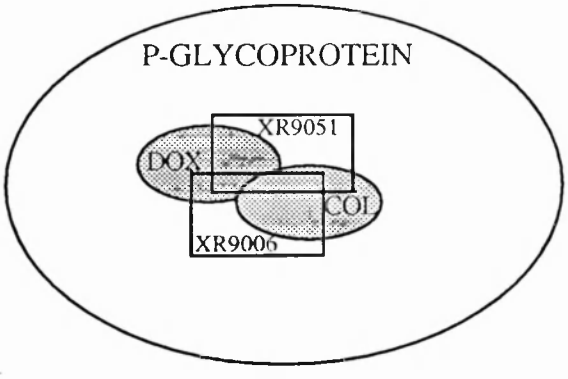
Interestingly, the only difference between XR9051 and XR9006 is that the side chain containing the tetrahydroisoquinoline group is in the meta position on the benzylidene ring in XR9051 and in the para position in XR9006. This difference in positioning of the tetrahydroisoquinoline group will serve to alter the conformation of the molecule. This may explain why two seemingly similar structures may have different activities. The two compounds may bind to slightly different sites on P-glycoprotein. If we assume that cytotoxics drugs bind to more than one binding site on P-glycoprotein and that resistance modifiers work (at least in part) by binding competitively to these binding sites we may hypothesise that the differential activity of the XR compounds may be dependent on where they bind to P-glycoprotein. For example XR9051 is a better modifier of doxorubicin resistance than colchicine resistance whereas XR9006 is a better modifier of colchicine resistance than doxorubicin. This may indicate that XR9051 binds to P-glycoprotein at or in very close proximity to the doxorubicin binding site and XR9006 binds to P-glycoprotein at or near the colchicine binding site. Figure 4.10 is a simplified diagram to illustrate this point. The circle represent drug binding sites and the squares XR compound binding sites. The XR9051 binding site overlaps with the doxorubicin site to a greater extent than the colchicine site. In contrast, the XR9006 binding site overlaps with the colchicine site to a greater extent than the doxorubicin site. The detailed structure of P-glycoprotein is not shown.

Safa et al. (1985) suggested that theoretically a competitive antagonist should not affect the dissociation rate constant of another drug. As such an antagonist can only bind when the bound drug has dissociated from the receptor site. Ferry *et al.* (1992) found that several 1,4-dihydropyridine analogues and other modifiers of P-glycoprotein-mediated MDR, including verapamil, increased the dissociation constant of [^{3}H]vinblastine indicating that they must be acting at a site distinct from the vinblastine-selective site. The group introduced the theory that drug binding domains on P-glycoprotein may be topographically distinct and allosterically coupled. If this

theory is correct it is possible that the XR9051 and XR9006 bind to separate (possibly overlapping) drug binding sites which are allosterically coupled so that drug binding to one site causes a conformational change to the other site resulting in an alteration of the drug specificity of that site.

Figure 4.10

A hypothetical representation of the interaction between the XR compounds XR9051 and XR9006 and the drugs doxorubicin and colchicine.



DOX-doxorubicin; COL-colchicine

4.4.3 Structure-toxicity relationships

It is very difficult to measure the intrinsic toxicity of a drug without looking in more detail at the metabolism and kinetics of the agent. There are, however, several features of the 10 compounds studied here which may explain their toxicity profile. As mentioned above, the only difference between XR9051 and XR9006 is the position of the side chain containing the tetrahydroisoquinoline group. In XR9006 the group is in the para position on the benzylidene ring whereas in XR9051 it is in the meta position. XR9006 is more toxic than XR9051 and this may be due to the fact that the para positioning of the tetrahydroisoquinoline group means that it is more likely to be exposed and therefore may be more prone to amide hydrolysis by proteases. The substituted analine (NH_2 group attached to an aromatic ring) that

results from such hydrolysis is known to have toxic properties. The most toxic molecule XR9112 has an ethyl group attached to the nitrogen in the 5 position of the diketopiperazine ring. XR9112 is identical to XR9006 apart from the fact that XR9006 has a methyl group attached to the nitrogen in the 5 position of the diketopiperazine ring. The addition of alkyl groups to the molecule increases the lipophilicity. XR9112 is likely to be more lipophilic than XR9006 and therefore more membrane permeable. It may reach higher concentrations within the cell. This explains why XR9112 is more potent at lower doses and also why it is toxic at higher doses. The lipophilicity of the molecule may indicate that at high doses the molecule accumulates to a large degree in the cell membrane. This may affect the integrity (i.e. fluidity and rigidity) of the membrane. Compounds XR1779 and XR1818 are very lipophilic and have no positive charge at physiological pH. The toxicity of these compounds may also be due to the fact that they are likely to accumulate to a large degree in the cell membrane thus disrupting its integrity. The addition of alkyl groups increases the occurrence of steric interactions within the molecule and therefore causes changes in molecular conformation. These conformational changes may have important implications in terms of toxicity and activity. A molecule may be a substrate for more than one receptor. A very slight change in molecular conformation may cause a molecule to change its affinity for each receptor. It is possible that the addition of an alkyl group (as in the case of XR9112) may cause a conformational change thereby causing the molecule to have a greater affinity for a receptor which triggers a toxic response.

4.4.4 Summary

It appears that within this series of compounds the presence of a diketopiperazine with a substituted tetrahydroisoquinoline group produces the most active compounds. XR9051 and XR9006 are the most active compounds. XR9051 is the best modifier of doxorubicin resistance and XR9006 is the best modifier of colchicine resistance. These data also confirm that molecules able to undergo protonation at physiological pH to form cationic species are the most effective modifiers of MDR. Lipophilicity is also important although extreme lipophilicity may also be the cause of toxicity due to alterations in membrane integrity. It also appears that the addition of alkyl groups also increases toxicity presumably by increasing lipophilicity.

Chapter 5

The discovery of XR9173 - a modifier of multidrug resistance-associated protein (MRP)-mediated multidrug resistance

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(ii) Effect of XR9173 on the accumulation of [3H]daunorubicin in the drug ensitive cell line, CEM and its P-glycoprotein-overexpressing subline, CEM/VLB

5.4 Discussion

5.4.1 Introduction

5.4.2 XR9173, structure-activity relationships

5.4.3 XR9173 as a modifier of MRP-mediated MDR

5.4.4 Other modifiers of MRP-mediated MDR

5.1 Introduction

5.1.1 The multidrug resistance associated protein (MRP)

The multidrug resistance associated protein (MRP) is the product of an ATP-binding cassette transporter gene overexpressed in some tumour cells resistant to antineoplastic agents. Section 1.3.3.iv describes some of the proposed mechanisms of action of MRP. It is clear that MRP induces ATP-dependent removal of drug from tumour cells. The mechanisms for this active transport have not been clearly defined. These mechanisms are discussed further in Chapter 6.

5.1.2 Reversal of MRP-mediated MDR

P-glycoprotein mediated MDR can be reversed *in vitro* by resistance modifying agents such as cyclosporin A, verapamil and PSC-833. These agents are all currently undergoing clinical trials in an attempt to overcome clinical drug resistance. These modifiers have not, so far, been shown to reverse MRP-mediated MDR to the same extent as P-glycoprotein-mediated MDR (Zaman *et al.*, 1994; Leier *et al.*, 1994a and b). Two important exceptions are the MRP-overexpressing HL60/ADR cell line where verapamil and PSC-833 are good inhibitors of drug transport (Feller *et al.*, 1995) and the MRP-overexpressing POGB/DX cell line where verapamil was able to reverse drug resistance and to increase drug accumulation (Binashi et al., 1995).

Most of the well-characterised MRP overexpressing cell lines have been selected for resistance to doxorubicin (Lutzky *et al.*, 1989; Cole *et al.*, 1992a & b; Krishnamachary *et al.*, 1993; Barrand *et al.*, 1994). Selection in doxorubicin has been shown to produce cells displaying a variety of resistance mechanisms. As well as overexpression of the membrane transport proteins, P-glycoprotein and MRP, other mechanisms such as a reduced topoisomersae II activity (Fernandes *et al.*, 1994) and detoxification of doxorubicin and doxorubicin-induced free-radicals by GSH (Tew, 1994) have been demonstrated in cells selected in doxorubicin. Lutzky

et al. (1989) found that depletion of cellular GSH by treating cells with the inhibitor of GSH synthesis, DL-buthionine S,R-sulfoximine (BSO), increased cellular daunorubicin accumulation and retention. More recently Versantvoort *et al.* (1995a) demonstrated that drug transport in MRP-overexpressing but not P-glycoprotein-overexpressing MDR cells can be regulated by cellular GSH levels. They found that BSO inhibited drug efflux from MRP, but not P-glycoprotein-mediated MDR cells and that this effect could be reversed by the restoration of intracellular GSH levels by treating the cells with GSH-ethylester which enters the cells and is then converted to GSH. This study also showed that BSO increases the toxicity of daunorubicin, vincristine and rhodamine 123 in a number of MRP-overexpressing tumour cell lines. Other modulators of MRP have been reported. Firstly, the isoflavonoid, tyrosine kinase inhibitor, genistein increases daunorubicin accumulation in several MRP but not P-glycoprotein-overexpressing MDR cell lines (Versantvoort *et al.*, 1993). The toxicity of genistein, however, limits its potential as a clinically useful resistance modifier. The second group of MRP modifiers are modulators of organic anion transport. Probenecid, typically used in the clinic to compete with penicillin for transport in the renal proximal tubule thereby increasing circulating penicillin levels, has been shown to modulate MRP-mediated MDR (Gollapudi *et al.*, 1995). In addition the leukotriene LTD_4 receptor antagonist, MK571 specifically modulates MRP-associated multidrug resistance (Gekeler *et al.*, 1995b). This is especially relevant in light of the observations of Jedlitschky *et al.* (1994) who demonstrated that MRP mediates the ATP-dependent transport of LTC_4 and structurally related anionic amphiphilic conjugates. Finally, the PKC inhibitor, GF 109203X (Gekeler *et al.*, 1995a) has been shown to affect MRP-mediated drug transport. It is not been determined whether this effect is due to an interaction (either non-competitive or competitive) with drug binding sites or with the ATP binding sites of MRP or affects MRP activity indirectly by altering its phosphorylation state. Melvin Center's group have recently demonstrated that MRP is highly phosphorylated and that the phosphate groups are metabolically

active and undergo cycles of phosphorylation and dephosphorylation in the cell. The protein kinase inhibitors H-7, staurosporine and chlereythrine can reduce phosphorylation and produce a concomitant inhibition of drug efflux in resistant cells (Ma *et al.*, 1995). These results may indicate that altering the phosphorylation state of MRP may affect its function.

5.1.2 Aims

The Xenova compounds are not exposed to a primary screen to evaluate their potential as modifiers of MRP-mediated resistance in the laboratories in Slough. In our laboratories all the compounds undergo assays involving the L23/P cell line and its resistant, MRP-overexpressing variant, L23/R (see Section 2.1). The aim of the experiments outlined in this chapter were to establish, (a) whether any of the XR compounds we received in Cambridge are modifiers of MRP-mediated resistance, (b) the extent of their modifying capacity, and (c) if XR compounds that act as MRP modifiers are also able to reverse P-glycoprotein-mediated MDR. We did not set out to compile a set of structure activity relationships for all of the new MRP test compounds but to elucidate the most active compounds and examine the activity of these compounds in more detail.

5.2 Materials and Methods

5.2.1 Cell lines

For studies involving MRP we used the human large cell lung cancer cell line, L23/P and the adenocarcinoma line MOR/P together with their respective MRP-overexpressing variants L23/R and MOR/0.4R. For studies involving P-glycoprotein we used the human T-lymphoblastoid cell line CEM together with its P-glycoprotein overexpressing variant, CEM/VLB. Details of the derivation, maintenance and culture conditions of these cell lines are described in Section 2.1.

5.2.2 Drug sensitivity testing

The cytoxicity of the XR compounds and evaluation of their ability to sensitise the MRP-overexpressing resistant cell lines, L23/R, MOR/0.4R and the P-glycoprotein overexpressing cell line CEM/VLB were determined using the tetrazolium (MTT) reduction assay. This method is described in detail in Section 2.3.

5.2.3 Drug accumulation studies

The effect of the XR compounds on the ability of L23/R cells to accumulate [^{3}H]daunorubicin and [^{3}H]colchicine was determined as described in Section 2.5.1.i

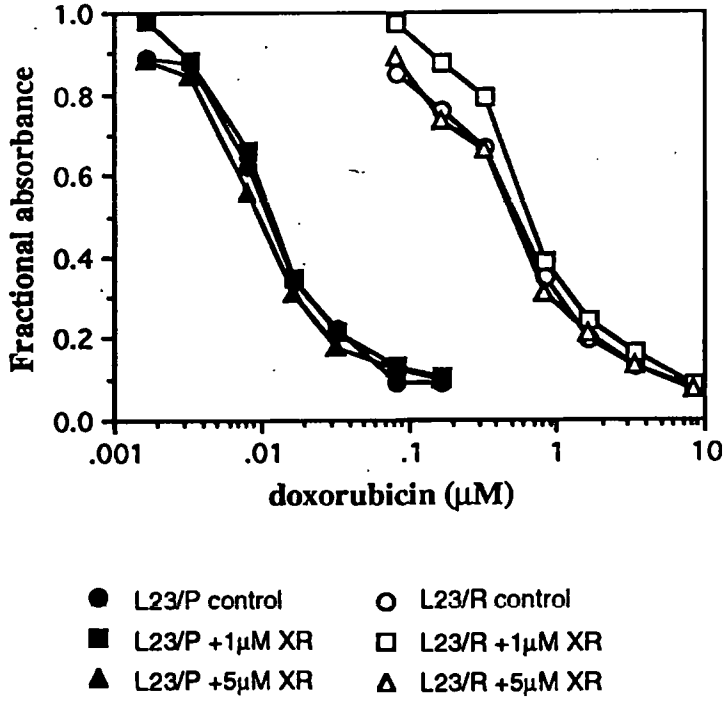
5.3 Results

5.3.1 XR9089 reverses resistance in COR-L23/R, an MRP-overexpressing MDR cell line

All of the novel fungal products from Xenova were routinely screened not only for their ability to modify P-glycoprotein-mediated MDR but also MRP-mediated MDR. Figure 5.1 demonstates the effect of all 10 compounds on the sensitivity of L23/P and L23/R to doxorubicin. Compounds, XR1824, XR1818 and XR9112 were toxic at 5μM in this system. Only the results at 1μM are shown on these graphs.

Figure 5.1

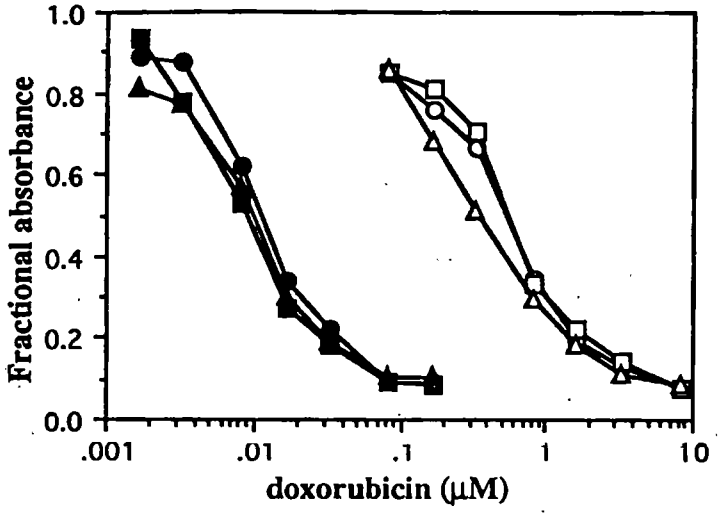
(i) Effect of XR1500 on the sensitivity of L23/P and L23/R cells to doxorubicin



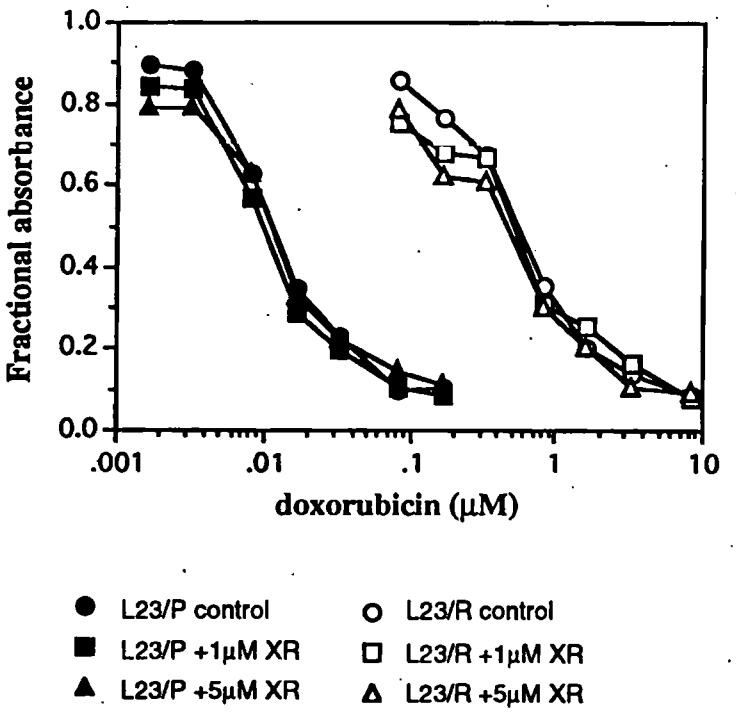
Graphs represent typical data sets. Similar results were obtained in 2 independent experiments. Fractional absorbance is defined by mean optical denisity of treated group divided by that of the control group. Each value represents the mean fractional absorbance in 4 replicate wells. Variation in fractional absorbance between replicate wells was generally less than 10%.

Figure 5.1 (continued)

(ii) Effect of XR1779 on the sensitivity of L23/P and L23/R cells to doxorubicin



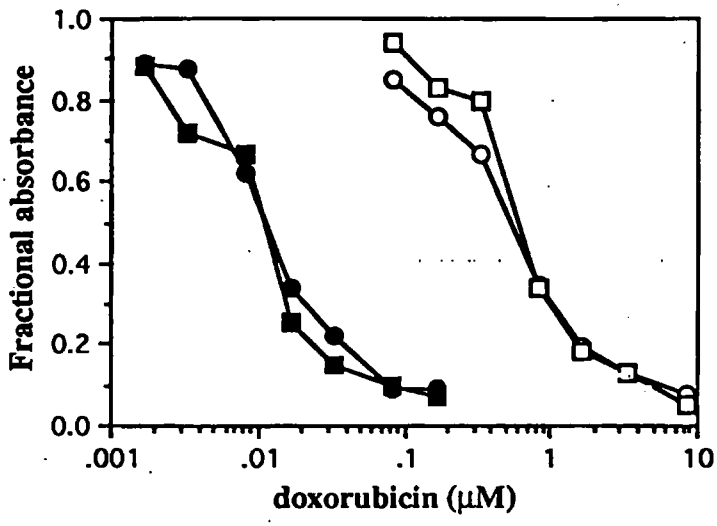
(iii) Effect of XR9051 on the sensitivity of L23/P and L23/R cells to doxorubicin



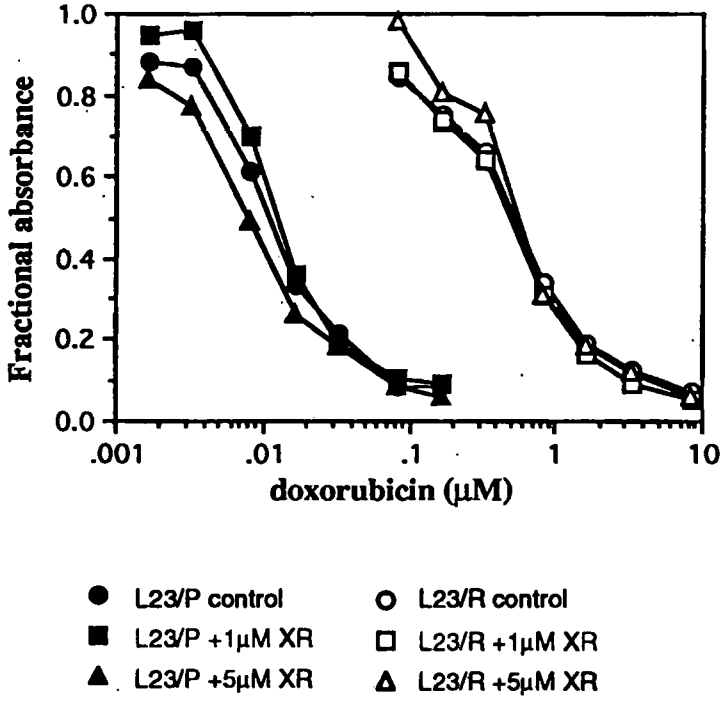
Graphs represent typical data sets. Similar results were obtained in 2 independent experiments. Fractional absorbance is defined by mean optical denisity of treated group divided by that of the control group. Each value represents the mean fractional absorbance in 4 replicate wells. Variation in fractional absorbance between replicate wells was generally less than 10%.

Figure 5.1 (continued)

(iv) Effect of XR1824 on the sensitivity of L23/P and L23/R cells to doxorubicin



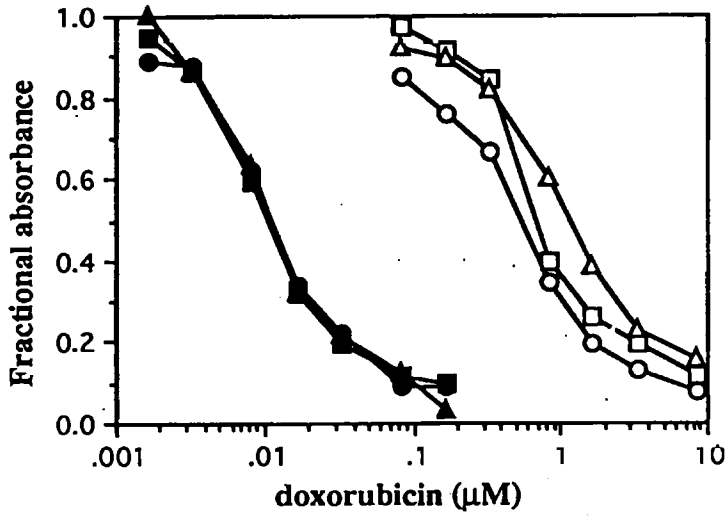
(v) Effect of XR1829 on the sensitivity of L23/P and L23/R cells to doxorubicin



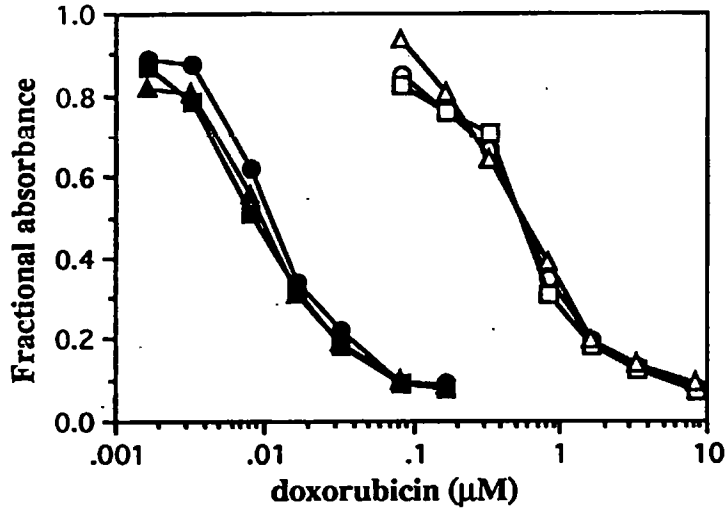
Graphs represent typical data sets. Similar results were obtained in 2 independent experiments. Fractional absorbance is defined by mean optical denisity of treated group divided by that of the control group. Each value represents the mean fractional absorbance in 4 replicate wells. Variation in fractional absorbance between replicate wells was generally less than 10%.

Figure 5.1 (continued)

(vi) Effect of XR9006 on the sensitivity of L23/P and L23/R cells to doxorubicin



(vii) Effect of XR9019 on the sensitivity of L23/P and L23/R cells to doxorubicin

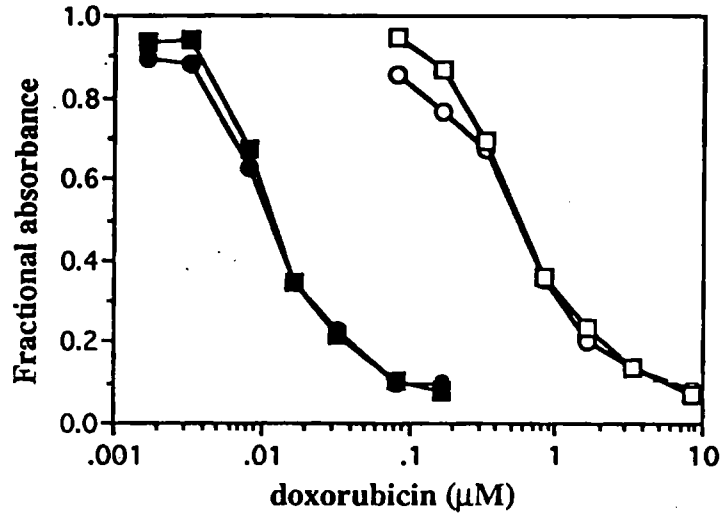


● L23/P control ○ L23/R control
■ L23/P +1μM XR □ L23/R +1μM XR
▲ L23/P +5μM XR △ L23/R +5μM XR

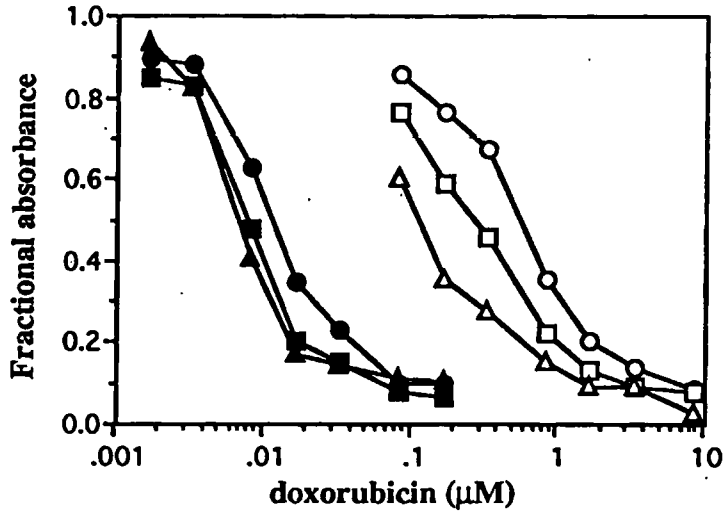
Graphs represent typical data sets. Similar results were obtained in 2 independent experiments. Fractional absorbance is defined by mean optical denisity of treated group divided by that of the control group. Each value represents the mean fractional absorbance in 4 replicate wells. Variation in fractional absorbance between replicate wells was generally less than 10%.

Figure 5.1 (continued)

(viii.) Effect of XR1818 on the sensitivity of L23/P and L23/R cells to doxorubicin



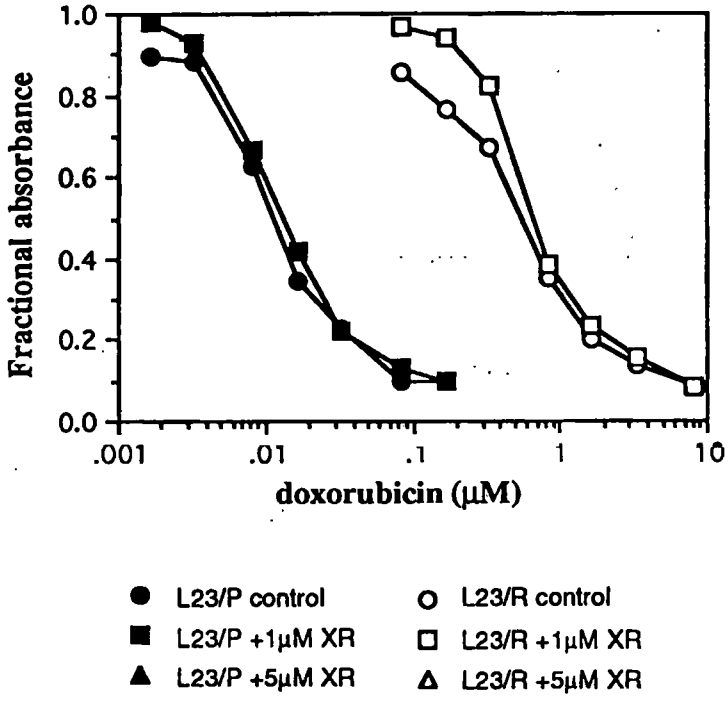
(ix) Effect of XR9089 on the sensitivity of L23/P and L23/R cells to doxorubicin



Graphs represent typical data sets. Similar results were obtained in 2 independent experiments. Fractional absorbance is defined by mean optical denisity of treated group divided by that of the control group. Each value represents the mean fractional absorbance in 4 replicate wells. Variation in fractional absorbance between replicate wells was generally less than 10%.

Figure 5.1 (continued)

(x) Effect of XR9112 on the sensitivity of L23/P and L23/R cells to doxorubicin



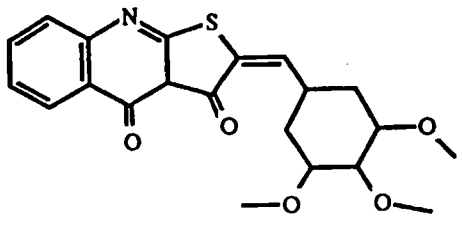
Graphs represent typical data sets. Similar results were obtained in 2 independent experiments. Fractional absorbance is defined by mean optical denisity of treated group divided by that of the control group. Each value represents the mean fractional absorbance in 4 replicate wells. Variation in fractional absorbance between replicate wells was generally less than 10%.

Of the initial batch of 10 compounds selected for detailed study only one compound, XR 9089, showed any activity versus MRP-mediated MDR. XR9089 at 1 and 5μM was able to sensitise the resistant cell line, L23/R, to doxorubicin by factors of 2 and 4 respectively (Figure 5.1.ix). This initial observation then lead to a derivatisation programme at Xenova. Ten new compounds were synthesised from XR 9089 (Figure 5.2). These compounds were then tested in our laboratory and in the laboratories at Xenova for their ability to: (a) reverse the accumulation deficit in L23/R cells and (b) sensitise L23/R cells to doxorubicin. We also examined their ability to modify P-glycoprotein mediated MDR.

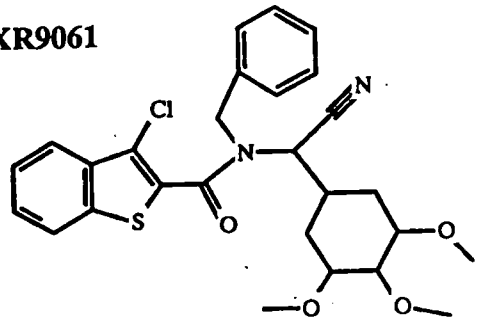
Figure 5.2

Structure of test MRP compounds

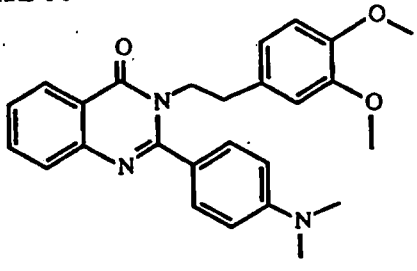
XR9032



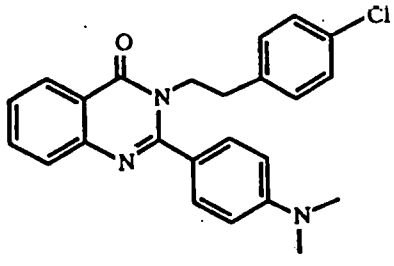
XR9061



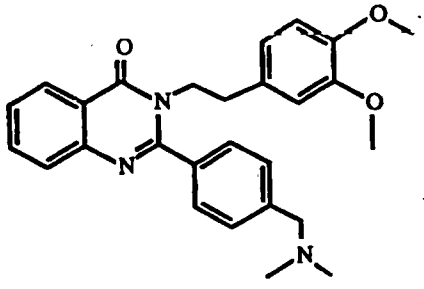
XR9062.1



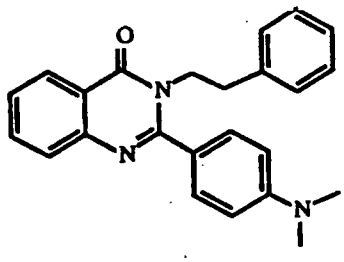
XR9124



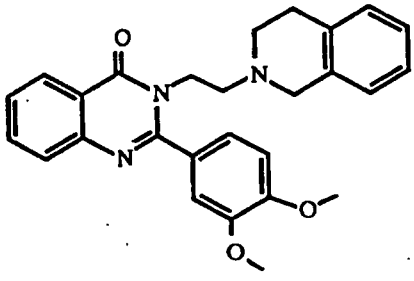
XR9142



XR9143



XR9152



XR9167

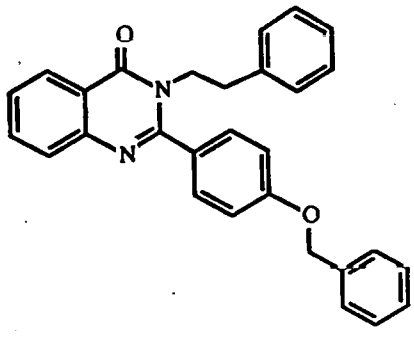
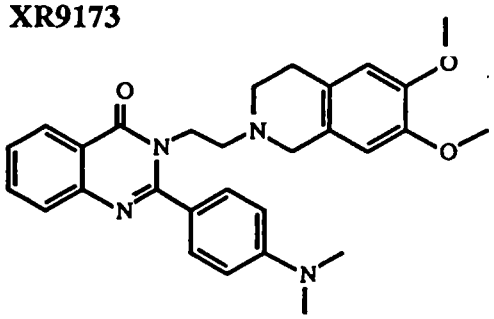


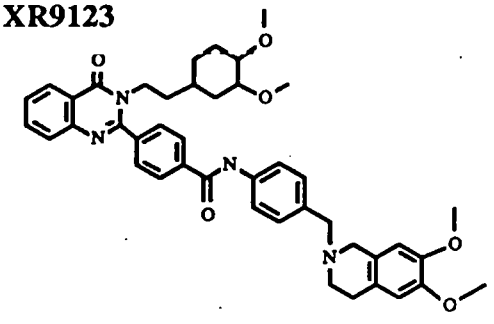
Figure 5.2 (continued)

Structure of test MRP compounds

XR9173



XR9123

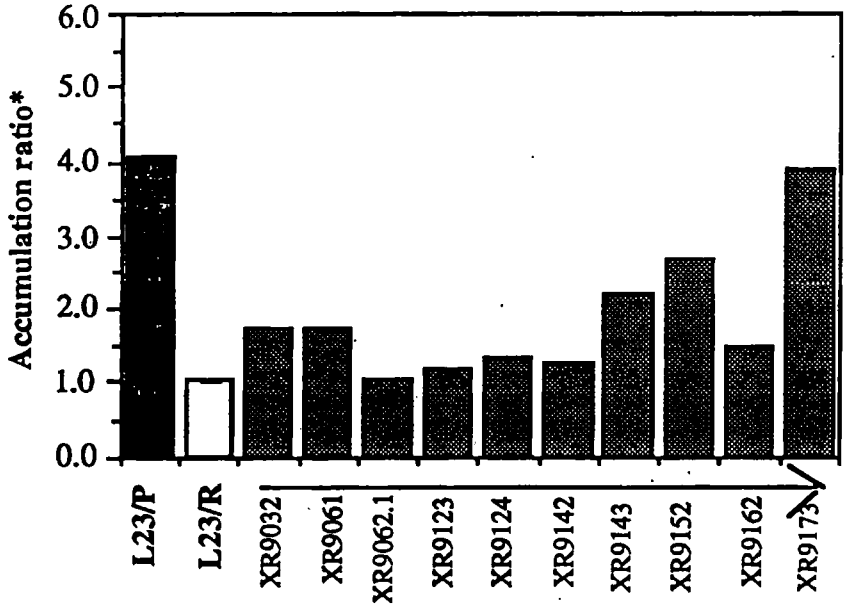


5.3.2. Effect of novel XR9089 derivatives on the accumulation of [^{3}H]daunorubicin in L23/R cells

On their arrival in Cambridge, all 10 of the test MRP XR compounds were examined for their ability to reverse the accumulation deficit in L23/R, MRP-overexpressing cells. Figure 5.3 demonstrates the difference in the ability of L23/P and L23/R cells to accumulate [^{3}H]daunorubicin. Table 5.1 shows the independent accumulation ratio values for 2 independent experiments.

Figure 5.3

Effect of test MRP XR compounds (5μM) on the accumulation of [^{3}H]daunorubicin in L23/R cells.



*Accumulation ratio is defined by the ratio of daunorubicin accumulation in the presence/absence of modifier. In the case of L23/P accumulation ratio is represented by daunorubicin accumulationin L23/P divided by daunorubicin accumulation in L23/R.

Values represents mean of 2 independent experiments.

Accumulation ratios for the 2 independent experiments are given in table 5.1.

Table 5.1

Effect of test MRP compounds (5μM) on the accumulation of [^{3}H]daunorubicin in L23/R cells

GROUP	Accumulation ratio
P/R ratio	4.57 3.54 (4.05)
Control	1.00 1.00 (1.00)
9032	1.55 1.93 (1.74)
9061	1.82 1.65 (1.73)
9062.1	0.72 1.30 (1.01)
9123	0.94 1.41 (1.17)
9124	1.25 1.40 (1.32)
9142	1.13 1.37 (1.25)
9143	2.09 2.27 (2.18)
9152	3.12 2.21 (2.66)
9167	1.62 1.31 (1.46)
9173	4.69 3.05 (3.87)

*Accumulation ratio is defined by the ratio of daunorubicin accumulation in the presence/absence of modifier. Figures represent individual values, means of 2 values are shown in parentheses. P/R ratio is defined by the ratio of daunorubicin accumulation in the parent/resistant cell line.

All the compounds modestly increased the accumulation of [^{3}H]daunorubicin in L23/R cells. Compounds XR9173, XR9143 and XR9152 all had mean accumulation ratios of greater than 2 at a concentration of 5μM. XR9173, however, was clearly the best compound in terms of reversing the accumulation deficit in L23/R cells. The compound caused accumulation to be increased by a factor of 4 compared to the control value (accumulation in the absence of modifier) bringing accumulation levels in the resistant cell line almost to those observed in the parent line (in the absence of the modifier).

At this stage, we decided to continue experimentation with only 3 of the MRP XR compounds. We chose the 3 compounds which produced the largest reversal of accumulation deficit in L23/R cells. It is clear from figure 5.3. that these candidate compounds were XR9173, XR9143 and XR9152 which had accumulation ratios of 3.9, 2.2 and 2.7 respectively. We then went on to evaluate, in greater detail, the ability of these 3 compounds to modify MRP-mediated MDR.

5.3.3 Effect of XR9173, 9143 and 9152 on the sensitiation of L23/R cells to doxorubicin

Firstly, we examined the effect of the 3 MRP XR compounds (MRP XRs) on the sensitivity of L23/R cells to doxorubicin. Figure 5.4 demonstrates that the sensitisation data supports the accumulation data shown in figure 5.3. XR9173 was clearly the most effective of the 3 MRP XRs in terms of reversing the accumulation deficit in L23/R cells and in sensitising the drug resistant L23/R cells to doxorubicin. We used the non toxic doses of 5 and 10μM. Toxicity studies performed in Cambridge and at Xenova confirmed that the IC_{50}s of XR9173, XR9143 and XR9152 were greater than 20μM (data not shown). For all three compounds there was little difference in the sensitisation observed on addition of either 5 or 10μM of MRP XR. This may suggest that whatever mechanism is responsible for this reversal of drug resistance in this cell line is saturable. These

data suggest that XR9173 is an efficient modifier of MRP mediated MDR. To confirm this observation, we decided to examine the potential of XR9173 to modify resistance in another MRP-overexpressing cell line, MOR/0.4R. In addition we examined its ability to sensitise both L23/R and MOR/0.4R to a number of different cytotoxic drugs.

Figure 5.4

(a) Effect of XR9173 on the sensitisation of L23/R cells to doxorubicin

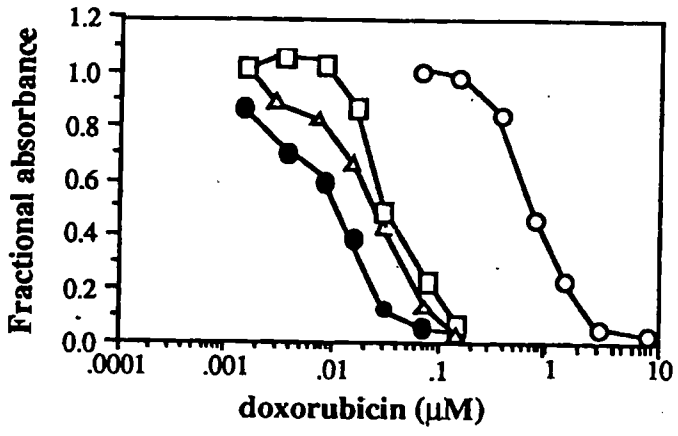
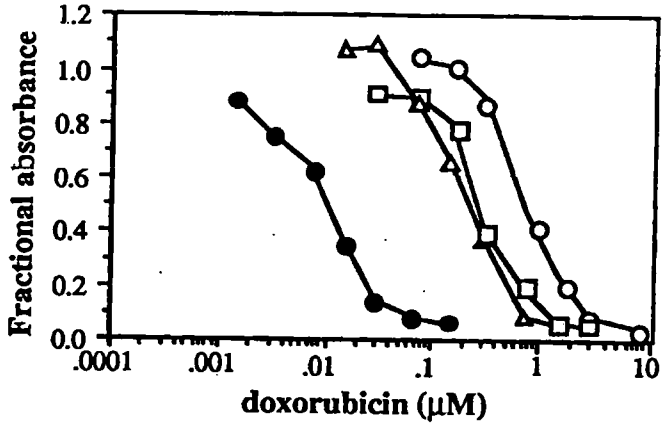


Figure 5.4

(b) Effect of XR9143 on the sensitisation of L23/R cells to doxorubicin



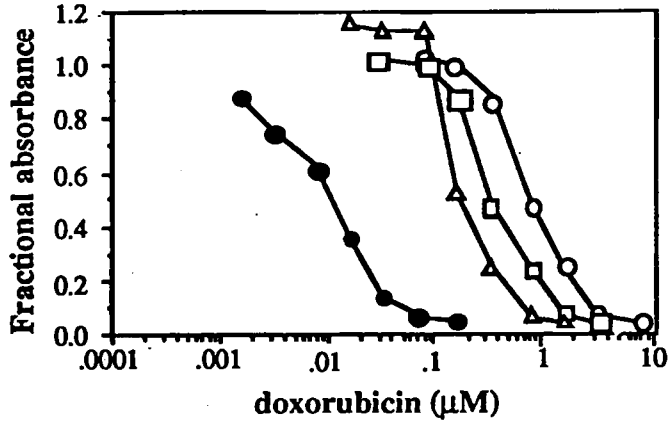
Graphs represents typical data sets.

Similar results were obtained in a at least 3 independent experiments.

(●) parent,L23/P cells, (○) resistant, L23/R cells, (□) L23/R + 5μM 9173, (▲) L23/R + 10μM 9173

Figure 5.4

(c) Effect of XR9152 on the sensitisation of L23/R cells to doxorubicin



Graphs represents typical data sets.

Similar results were obtained in at least 3 independent experiments.

(●) parent,L23/P cells, (○) resistant, L23/R cells, (□) L23/R + 5μM 9173, (▲) L23/R + 10μM 9173

5.3.4 Effect of XR9173 on the sensitisation of L23/P and MOR/P and their respective MRP-overexpressing MDR variants, L23/R and MOR/0.4R

The values in table 5.2 represent sensitisation ratios for XR9173 at two concentrations, 1μM and 5μM. Sensitisation ratio is defined by the IC_{50} for the relevant drug in the presence of XR9173, divided by the IC_{50} for the relevant drug in the absence of XR9173 (IC_{50} = concentration of cytotoxic drug required to reduce fractional absorbance to 50% of control values in the MTT assay).

When interpreting the data in table 5.2. it should be noted that the cell lines used are diffentially resistant to all three cytotoxic drugs. For example in the case of the L23 cell lines; the resistant, MRP overexpressing, L23/R is 20- to 30- fold resistant to doxorubicin, 10- to 20-fold more resistant to colchicine and only 5- to 10-fold resistant to taxol compared to the sensitive L23/P cell line (Table 5.3). The resistance factors for MOR/0.4R also differ depending on the cytotoxic drug to

which they are exposed. Resistance factor is defined by the IC_{50} of the drug in the resistant line divided by the IC_{50} of the drug in the sensitive line.

Table 5.2

Effect of XR9173 on the sensitisation of L23/P and MOR/P and their respective MRP-overexpressing MDR variants, L23/R and MOR/0.4R

	Sensitisation Ratio[1] DOXORUBICIN		Sensitisation Ratio[1] COLCHICINE		Sensitisation Ratio[1] TAXOL	
CELLS	9173 1μM	9173 5μM	9173 1μM	9173 5μM	9173 1μM	9173 5μM
L23/P	1.6 (0.2)	2.2* (0.2)	1.2 (0.1)	1.7 (0.3)	1.1 (0.1)	1.1 (0.1)
L23/R	5.1* (0.7)	17.4* (5.4)	4.5 (1.6)	8.5* (2.3)	1.3 (0.1)	2.4 (0.4)
MOR/P	1.6 (0.2)	3.3* (0.9)	1.1 (0.1)	1.5 (0.1)	1.2 (0.2)	1.2 (0.2)
MOR/0.4R	3.7 (1.2)	11.5* (2.0)	3.0 (0.5)	8.1* (0.9)	1.7* (0.9)	3.2** (0.2)

[1]Ratio of IC_{50} in presence/absence of modifier. *$p<0.05$(significantly different from 1), **$p<0.01$(highly significantly different from 1): Student's t test. Values are means of 3 independent experiments. Parentheses show standard error.. IC_{50} = dose of cytotoxic drug required to reduce fractional absorbance to 50% of control values in the MTT assay

Table 5.3 shows the differing resistance factors for these two cell lines and the three cytotoxic drugs, doxorubicin, taxol and colchicine.

Table 5.3

Resistance factors of doxorubicin, colchicine and taxol in the MRP-overexpressing cell lines L23/R and MOR/0.4R.

	Resistance factor* DOXORUBICIN	Resistance factor* COLCHICINE	Resistance factor* TAXOL
L23/R / L23/P	25.0	12.8	8.0
MOR/0.4R / MOR/P	32.2	14.8	5.8

Values are means of at least 2 independent experiments. *Resistance factor is defined by the IC_{50} of drug in resistant line divided by the IC_{50} of drug in the sensitive line.

XR9173 at a concentration of 5μM significantly increased the sensitivity of the drug sensitive, L23/P and MOR/P cell lines to doxorubicin.

In all other cases, XR9173 had no significant effect on the sensitivity of the drug sensitive cell lines to doxorubicin, colchicine or taxol (Student's t test). In the drug resistant cell lines, XR9173 at a concentration of 5μM significantly increased the sensitivity of the drug resistant cell lines, L23/R and MOR/0.4R to doxorubicin, colchicine and taxol. At 1μM, XR9173 was also signicantly increased the sensitivity of MOR/0.4R to colchicine and taxol. The ability of XR9173 to increase the sensitivity of MDR cell lines to cytotoxic drugs is clearly dependent on the cytotoxic drug used. From the values in table 5.2 we can see that L23/R cells are 25-fold resistant to doxorubicin, 12.8-fold more resistant to colchicine and 8-fold more resistant to taxol compared to the L23/P cells. XR9173 sensitises L23/R cells by 17.4-, 8.5- and 2.4-fold respectively to doxorubicin, colchicine and taxol. L23/R cells therefore have resistance factors in the presence of XR9173 (RF*) of 7.6, 4.3 and 5.6 repectively for doxorubicin, colchicine and taxol compared to L23/P cells. XR9173, therefore, produces a decrease in resistance factor for L23/R cells of 70%, 66% and 30% for doxorubicin, colchicine and taxol respectively. In

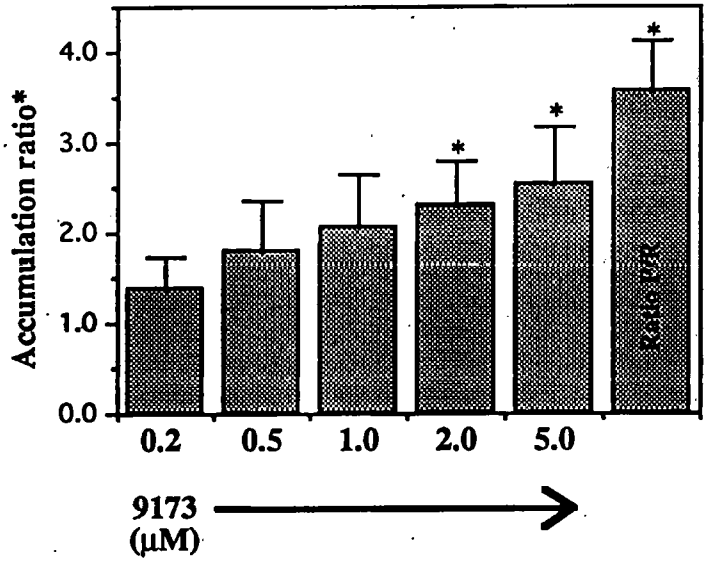
contrast, using the same evaluation method for MOR/0.4R cells, XR9173 produces a decrease in resistance factor of 36%, 30% and 56% for doxorubicin, colchicine and taxol respectively. It appears, therefore, that XR9173 is a more efficient modifier of taxol resistance than of doxorubicin and colchicine resistance in MOR/0.4 cells and a more efficient modifier of colchicine and doxorubicin resistance than of taxol resistance in L23/R cells. It appears, therefore, that the effect of XR9173 may also be cell specific.

5.3.5 Effect of XR9173 on the accumulation of [^{3}H]daunorubicin in L23/P and L23/R cells

We examined the effect of XR9173 on the accumulation of [^{3}H]daunorubicin and [^{3}H]colchicine in L23/R cells. Figure 5.5 a & b shows that XR9173 reversed the [^{3}H]daunorubicin and [^{3}H]colchicine accumulation deficit in L23/R, drug resistant cells in a dose dependent fashion. At a concentration of 5μM, full reversal was not achieved, although drug accumulation in the resistant cell line reached 71% and 86% of the parental levels for [^{3}H]daunorubicin and [^{3}H]colchicine respectively.

Figure 5.5

(a) Effect of XR9173 on the accumulation of [^{3}H]daunorubicin in L23/R cells



*p<0.05 (significantly different from 1.0).

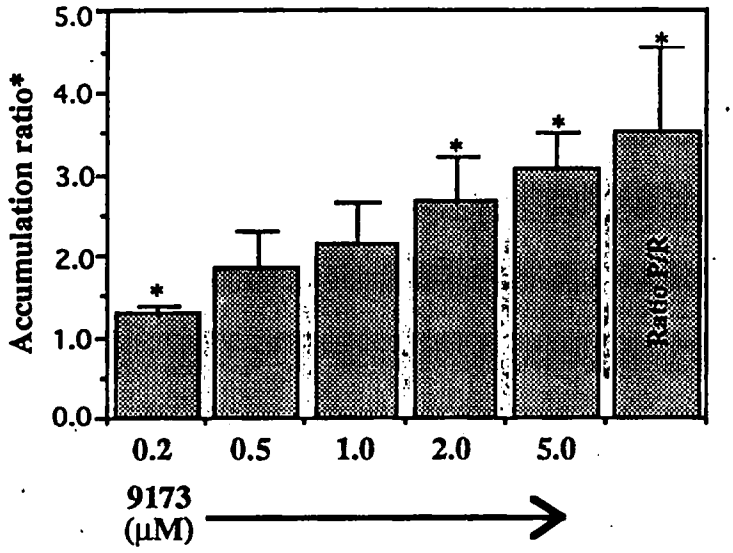
**p<0.01 (highly significantly different from 1.0, Student's t test)

Accumulation ratio =ratio of [^{3}H]drug accumulation in the presence/absence of modifier.

Values are means ± standard error of at least 3 independent experiments.

Figure 5.5

(b) Effect of XR9173 on the accumulation of [^{3}H]colchicine in L23/R cells



*p<0.05 (significantly different from 1.0).

**p<0.01 (highly significantly different from 1.0, Student's t test)

Accumulation ratio =ratio of [^{3}H]drug accumulation in the presence/absence of modifier.

Values are means ± standard error of at least 3 independent experiment.

5.3.6 Effect of XR9173 on P-glycoprotein mediated MDR

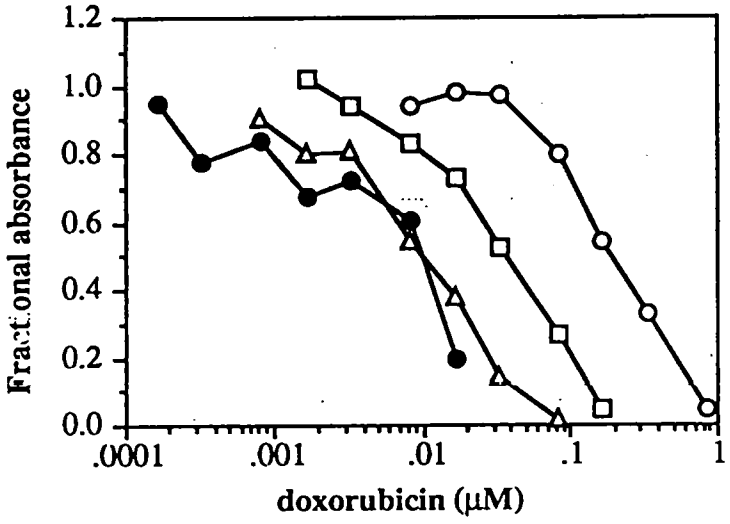
In order to establish whether or not XR9173 is also a modifier of P-glycoprotein-mediated MDR, we examined its activity in the drug sensitive cell line CEM together with the P-glycoprotein-overexpressing, MDR cell line, CEM/VLB.

(i) Effect of XR9173 on the sensitisation of the drug sensitive cell line, CEM and its P-glycoprotein-overexpressing subline, CEM/VLB to doxorubicin

XR9173 was not able to increase the sensitivity of the drug sensitive cell line , CEM to doxorubicin (data not shown). In the drug resistant cell line, CEM/VLB, XR9173 (5μM) restored sensitivity to doxorubicin to levels approaching that of the parent, drug sensitive cell line (Figure 5.6 & Table 5.4).

Figure 5.6

Effect of XR9173 on the sensitisation of CEM/VLB drug resistant cells to doxorubicin



Graph represents typical data sets. Similar results were obtained in 2 independent experiments.

Individual sensitisation ratios are shown in table 5.4

(●) parent,CEM cells, (○) resistant, CEM/VLB cells,

(□) CEM/VLB + 1μM 9173, (▲) CEM/VLB + 5μM 9173

Table 5.4

Effect of XR9173 (1&5μM) on the sensitisation of L23/R cells to doxorubicin

CELL LINE	Sensitisation Ratio (9173 1μM)	Sensitisation Ratio (9173 5μM)	Resistance Factor
L23/R	6.7 8.0	20.0 20.0	20.0 27.0

Values from 2 independent experiments are shown.

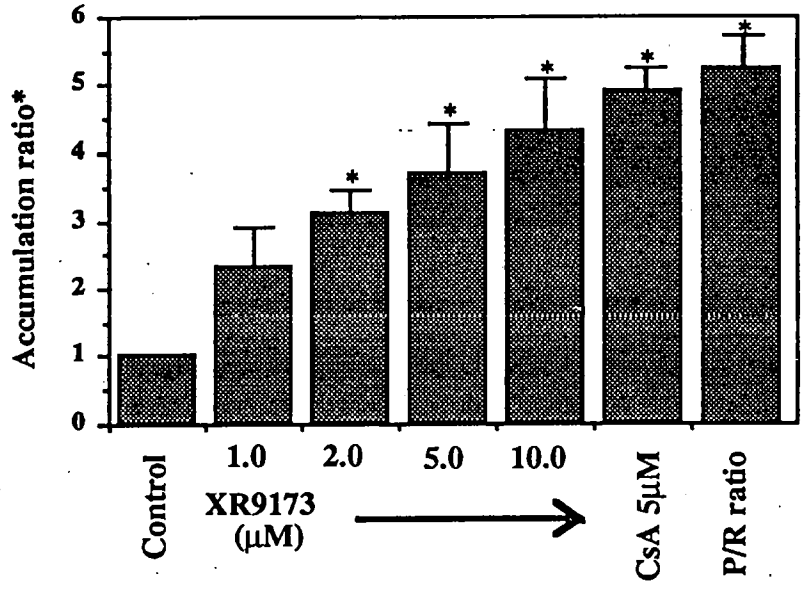
[1]Ratio of IC_{50} in presence/absence of modifier. IC_{50} = dose of cytotoxic drug required to reduce fractional absorbance to 50% of control values in the MTT assay. Resistance factor is defined by the IC_{50} of the drug in the resistant line divided by the IC_{50} of the drug in the sensitive line.

(ii) Effect of XR9173 on the accumulation of [^{3}H]daunorubicin in the drug sensitive cell line, CEM and its P-glycoprotein-overexpressing subline, CEM/VLB

XR9173 did not effect the levels of daunorubicin accumulation in the drug sensitive cell line, CEM (data not shown). However XR9173 was able to restore the accumulation deficit in the drug resistant, CEM/VLB, cell line. Figure 5.6 demonstrates that 10μM XR9173 caused a 4.5-fold increase in daunorubicin accumulation compared to control levels. In comparison 5μM cyclosporin A (a known modifier of P-glycoprotein-mediated MDR *in vitro*) increased daunorubicin accumulation by 5-fold compared to control levels. It appears, therefore, that XR9173 is almost equivalent to cyclosporin A as a modifier of P-glycoprotein-mediated MDR. Therefore, over a period of 1 h, both cyclosporin A and XR9173 restored the accumulation in the resistant cell line CEM/VLB cells to levels approaching that of the parental cell line, CEM.

Figure 5.7

Effect of XR9173 on the accumulation of [^{3}H]daunorubicin in the P-glycoprotein overexpressing subline, CEM/VLB (1 h exposure)



*Accumulation ratio =ratio of [^{3}H]drug accumulation in the presence/absence of modifier

Values are means of at least 3 independent experiments. Error bars indicate standard error.

P/R ratio=drug accumulation in drug sensitive cells divided by drug accumulation in drug resistant cells

5.4 Discussion

5.4.1 Introduction

At present, only very few reports exist on compounds that are able to reverse MRP-mediated MDR. The effectiveness of those compounds that have been found to have modest activity in MRP-overexpressing cell lines is limited either by their toxicity or by their lack of potency. An effective modifier of MRP-mediated MDR would prove to be a useful tool in the evaluation of mechanisms underlying the activity of MRP.

5.4.2 XR9173, Structure activity relationships

The data presented in here indicates that XR9173 is an efficient modifier of both P-glycoprotein- and MRP-mediated MDR. Figure 5.2 shows that XR9173 has a tetrahydroisoquinoline group within its structure. We demonstrated in Chapter 4 that the presence of this group within a molecule tends to increase its activity as a modifier of P-glycoprotein mediated MDR, possibly by increasing the positive charge of the molecule at physiological pH. Common properties of modulators of P-glycoprotein-mediated MDR are lipophilicity and the ability to be protonated at physiological pH (i.e. be cationic). XR9173 is both lipophilic and cationic, it is, therefore, not surprising that the compound is an active modifier of P-glycoprotein-mediated MDR. It is much more difficult to provide a simple explanation for the activity of XR9173 as a modifier of MRP-mediated MDR. As MRP is thought to be identical to the GSH conjugate transporter (Jedlitschky *et al.*, 1994) and several groups have reported ATP-dependent transport of glutathione *S*-conjugates it is possible that XR9173 interacts with this transport system. We know that MRP-overexpressing cells accumulate less daunorubicin and colchicine than their related parental cell lines and that XR9173 restores this accumulation deficit to levels approaching that of the parental, drug sensitive cells. It may, therefore, be possible that XR9173 interacts with the mechanism(s) involved in the transport of cationic drugs which may well be distinct from the GSH conjugate transporter.

5.4.3 XR9173 as a modifier of MRP-mediated MDR

The results show that of the ten compounds used in the P-glycoprotein study, only one, XR9089, was able to sensitise L23/R, MRP overexpressing cells to doxorubicin. It is clear that an efficient modifier of P-glycoprotein-mediated MDR is not necessarily a good modifier of MRP-mediated MDR. This is in agreement with the work of Barrand *et al.* (1993) who concluded that the mechanisms responsible for resistance in L23/R are relatively insensitive to the effects of modifiers that alter drug accumulation in P-glycoprotein mediated MDR cells, for example, cyclosporin A, PSC-833 and verapamil. Of the ten derivatives of XR9089, XR9173 was clearly the most effective modifier of MRP both in terms of reversing accumulation deficit and sensitising resistant cells lines to cytotoxic doxorubicin. The ability of XR9173 to increase the sensitivity of MDR cell lines to cytotoxic drugs is clearly dependent on the cytotoxic drug used. XR9173 is more efficient at sensitising the MRP-overexpressing cell line, L23/R, to doxorubicin and colchicine than to taxol. Conversely, XR9173 is more efficient at sensitising the MRP-overexpressing cell line, MOR/0.4R to taxol than it is doxorubicin and colchicine. The effects of XR9173 appear, therefore, to be both dependent on the cytotoxic drug and the cell line.

The results presented here show a discrepancy between the ability of XR9173 to increase drug accumulation in drug resistant cells and sensitise drug resistant cells to cytotoxic drugs. For example in L23/R cells, XR9173 is able to increase the accumulation of [^{3}H]colchicine to 86% of the parental level but only sensitises the resistant line to 66% of the parental level. Interestingly, these discrepancies between the chemosensitising effects of verapamil and its modulatory influence on drug accumulation have also been noted for P-glycoprotein (Keizer *et al.*, 1989). These discrepancies may indicate that drug accumulation over the course of a few hours is not a good guide to the effects of the same drug over a longer term survival assay. Keizer *et al.* also demonstrated similar levels of drug uptake in

variants of a human squamous lung cancer cell line despite 4-fold differences in their sensitivity to doxorubicin. It is possible that redistribution of drug away from sensitive target sites as opposed to overall decreased cellular accumulation of drug may be a significant factor in determining drug resistance.

Our results indicate that XR9173 is also an efficient modifier of P-glycoprotein mediated MDR. A compound able to modify more than one type of MDR *in vitro* may be of potential use in the clinic as clinical drug resistance is thought to be caused by more than one mechanism.

5.4.4 Other modifiers of MRP-mediated MDR

The first compound shown to enhance the decreased drug accumulation in an MRP-overexpressing cell line without affecting P-glycoprotein-mediated MDR cells was the isoflavonoid, tyrosine kinase inhibitor, genistein (Versantvoort *et al.*, 1993). The group showed that genistein was able partially to reverse the daunorubicin accumulation deficit and completely reverse the VP-16 accumulation deficit in the MRP-overexpressing GLC4/ADR MDR cell line. They suggest that genistein may bind directly to the MRP transporter and be pumped out of the cell. Any potential clinical use of genistein is limited by its cytotoxic effects. Recently, Gekeler *et al.* (1995a) showed that MRP associated MDR of HL60/AR and GLC4/ADR cells is efficiently modulated by the selective bisindolylmaleimide PKC inhibitor, GF 109203X. It remains unclear whether this compound acts via a direct interaction with MRP or via another mechanism involving the inhibition of PKC. Gekeler *et al.* (1995b) have also recently demonstrated that MK571, the leukotriene LTD_4 receptor antagonist, partially modulates MRP-mediated MDR. This result together with the demonstration that leukotriene LTC_4, the GSH conjugate of LTA_4, is transported in an ATP-dependent manner into membrane vesicles prepared from mouse mastocytoma cells (Leier *et al.* 1994a), from MRP overexpressing multidrug resistant HL60/ADR cells (Jedlitschky *et al.* 1994), or from HeLa MRP

transfectants (Leier et al 1994b) supports the evidence for a close relationship, if not identity, of the 190kDa MRP drug transporter with a GSH conjugate transporter. This is link between MRP and the glutathione conjugate transporter is discussed in greater detail in Chapter 6.

Barrand *et al.* examined the effect of some modifiers of P-glycoprotein-mediated MDR (Barrand *et al.* 1993) on L23/R, a cell line now known to overexpress MRP and used extensively in the experiments carried out in chapters 5 and 6 of this study. The group found that although cyclosporin A, PSC-833 and verapamil were all able to at least partialluy restore the daunorubicin and vincristine accumulation deficit in L23/R cells. Despite this all three modifiers produced only a modest degree of sensitisation to vincristine and daunorubicin. Verapamil at a concentration of 3.3μg/ml increased the accumulation of vincristine in L23/R cells to levels exceeding that of the drug sensitive cell line, L23/P. In contrast, however, the same dose of verapamil was not capable of restoring the sensitivity of the resistant cell line to that of the parent. This agrees with the results obtained with XR9173. Where the almost complete reversal of accumulation deficit did not correspond to the only partial sensitisation of the resistant cell line we observed.
In summary, of the original 10 derivatives of XR9089 tested for their ability to modifiy MRP-mediated MDR, XR9173 is the most active. The effects of XR9173 appear to be both cell line and drug specific. The discovery of this novel modifier of MRP-mediated MDR provides us with a useful tool for the investigation of the mechanism(s) underlying this type of resistance.

Chapter 6

XR9173
A Mechanistic Study

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6.5 Discussion

6.1 The proposed mechanisms of the multidrug resistance-associated protein (MRP)

6.1.1 MRP as a plasma membrane drug efflux pump

The multidrug resistance-associated protein (MRP) is a 190kDa membrane protein associated with resistance of human tumour cells to cytotoxic drugs. Transfection experiments have demonstrated that the MRP gene is a drug resistance gene (Grant *et al.*, 1994). These types of study do not, however, provide any clues as to the mechanism of action of MRP. As MRP belongs to the ABC superfamily of transporter proteins (Cole *et al.*, 1992a and b; Higgins *et al.*, 1992), it is possible that it may function as a plasma membrane drug efflux pump, similar to P-glycoprotein. In support of this, decreased drug accumulation has been reported for several non-P-glycoprotein cell lines that were later discovered to overexpress MRP (Zijlstra *et al.*, 1987; Zaman *et al.*, 1993; Barrand *et al.*, 1994). In contrast , however, the MDR H69AR cell line in which the *MRP* gene was cloned does not possess a drug accumulation deficit (Cole *et al.*, 1992a & b). In addition the subcellular location of MRP did not seem to be similar to that of a plasma membrane transporter such as P-glycoprotein. A 190kDa protein, thought to be MRP was found to be located on the endoplasmic reticulum rather than the plasma membrane (Marquardt *et al.*, 1990; Krishnamachary *et al.*, 1993). From studies using the SW-1573, human lung carcinoma cell line stably transfected with an expression vector containing *MRP* cDNA, Zaman *et al.* (1994) concluded that MRP acts as a drug efflux pump, similar to P-glycoprotein, extruding hydrophobic compounds from the cells against a concentration gradient. These conclusions were based on the following pieces of evidence: (a) Like P-glycoprotein, MRP can cause resistance to a broad spectrum of mainly hydrophobic drugs; (b) MRP is predominantly located on the plasma membrane (this is in contrast to the findings of other groups); (c) MRP can decrease drug accumulation in the cell and this decrease is abolished by plasma membrane permeabilisation and finally; (d) MRP can increase the efflux of drugs from cells. Zaman's group have therefore proposed a

model for MRP activity whereby the two ATP-binding motifs in MRP allow the protein, like P-glycoprotein, to use ATP hydrolysis to fuel an active transport efflux mechanism.

6.1.2 MRP as a transporter of glutathione-*S* -conjugates

Several group have recently reported ATP-dependent transport of Glutathione *S* -conjugates by the multidrug resistance associated protein (Jedlitschky *et al.*, 1994; Muller *et al.*, 1994). Leier *et al.* (1994a) demonstrated that photoaffinity labelling of mastocytoma cell membranes with LTC_4, the GSH conjugated leukotriene C_4, specifically labels a 190-kDa plasma membrane protein. This is the molecular weight of MRP. This group have also demonstrated that MRP mediates the ATP-dependent transport of LTC_4 and structurally related conjugates in membrane vesicles from HeLa cells transfected with an MRP expression vector (Leier *et al.*, 1994b). They speculated that the LTC_4 transporter and MRP are infact the same protein. In agreement with this interpretation is the demonstration that MRP is present in many different types of tissue, including erythrocytes, like the GSH *S* -conjugate transporter (Zaman *et al.*, 1993). One observation that decreases the plausibility of this model is that MRP confers resistance to drugs, such as doxorubicin and vincristine, that are not known to undergo major modifications in cells, or at least no modification that would turn the drug into a substrate for the GS-X pump (Muller *et al.*, 1994). Several explanations have been suggested for the paradox that overexpression of MRP results in the transport of negatively charged molecules (LTC_4, DNP-GS) as well as transport of neutral or positively charged drugs (VP-16 and doxorubicin): (a) negatively charged complexes may be formed in the cell, but due to instability they may not be detected. If the conjugated drug was unstable, it would be successively transported and then immediately split in the extra cellular medium into GSH and cytotoxic drug. It would be expected, therefore, that the export of drugs from inside MRP overexpressing MDR cells would lead to a rise in extra cellular GSH levels. Versantvoort *et al.* (1995a)

however, were not able to demonstrate any increase in GSH release after exposure of GLC4/ADR, MRP overexpressing, MDR cells to daunorubicin; (b) Awasthi *et al.* (1994) suggested that the GS-X pump may be more versatile than was originally thought and might therefore be able to transport both conjugated and unconjugated drugs; (c) it is possible that MRP may exist in two states functioning as a bifunctional transport protein for anionic GSH *S*-conjugates and cytotoxic drugs. An interesting interpretation of this model is one in which transport of GSH *S*-conjugates might induce a conformational change in MRP which enables the efflux of cytotoxic drugs. Modulation of the transport of GSH *S*-conjugates by GSH depletion would, therefore, affect the drug transport activity. This is supported by the fact that MRP-mediated drug transport can be regulated by GSH depletion with BSO (Versantvoort *et al.*, 1995a). Additionally, this model allows for inhibition of drug transport by agents other than those which regulate GSH, for example, genistein (Versantvoort *et al.*, 1994) (d) MRP might not be the GS-X pump but instead activate an endogenous GS-X pump. This explanation is rather unlikely due to the fact that LTC_4 has been shown to specifically label a 190kDa protein (as discussed above). Alternatively, MRP may itself be the GSH *S*-conjugate transporter which activates an endogenous latent drug transport protein.

For many years, there has been an association between drug resistance and raised cellular levels of GSH and glutathione -*S*-transferases (GSTs). To date the emphasis has been on detoxification of drugs and on the peroxidation products that some drugs generate. Efficient export of drug conjugates or cytotoxic peroxidation products from the cell may be essential to generate resistance to the continued presence of drug.

6.2 Glutathione (GSH)

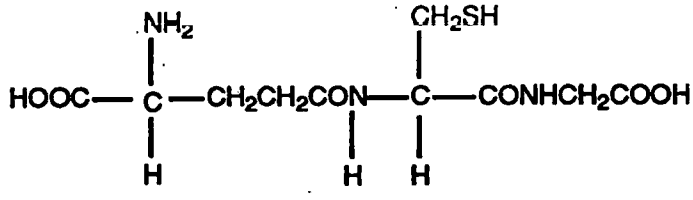
6.2.1 Chemical properties of GSH

It is clear that there is a link between cellular glutathione metabolism and the mechanism of action of MRP. It is important to understand the basics behind the metabolism of GSH and its cellular function with respect to the biology of cell membrane transport.

Glutathione (GSH) is the most important non-protein thiol in living systems, and is of widespread occurrence in the intracellular milieu of animals, plants and micro-organisms. GSH was isolated and named by the English biochemist Frederick Gowland Hopkins (1921). The chemical structure of GSH is given below in figure 6.1.

Figure 6.1

Structure of GSH



An understanding of the significance of GSH in the operation of cells has slowly evolved through studies on a variety of biological systems and biochemical reactions. It has recently become clear that GSH is involved in the regulation of transport of cytotoxic drug mediated by MRP. A better understanding of the biology and biochemistry of GSH function and metabolism may be the key to determining the mechanism(s) of action of MRP. The GSH status of cells is defined by the total cellular concentration of GSH and the nature of the distribution of the possible forms in which GSH can occur in cells. The most important forms of this compound include GSH itself, glutathione disulphides (GSSG) and mixed

disulphides, mostly GSS-protein. Other possibilities are thiol esters and GSH derivatives bound through linkages other than sulphur. In addition, hidden pools of GSH, in which GSH is available to some reactants, may exist (Palekar *et al.*, 1975). Other cellular components which behave chemically like GSH or GSSG, for example, cysteine or reactive disulphides, should be considered in any evaluation of the GSH status of a cell. GSH (MW 307.33) is a white crystalline solid which is freely soluble in water, dilute ethanol, liquid ammonia and dimethylformamide. The molecule possesses both acidic and basic groups.

6.2.2 Cellular function of GSH in cells and organelle membranes

The structural integrity of the cell membrane and the membranes of intracellular organelles depends upon a suitable level and normal distribution of the various forms of GSH within the cell. In this situation the GSH is involved in the regulation of thiol/disulphide ratio in proteins through interchange reactions, and protection of lipids against peroxide through the interception of free radicals and the decomposition of lipid hydroperoxides. Various membrane functions, including ion and sugar transport, mitochondrial functions, the release of neurotransmitters and the action of hormones, are apparently dependent upon a suitable thiol-disulphide balance within the cell which can be altered by a change in the GSH status of the cell or system. For example, in the lens, oxidation of a portion of the GSH is accompanied by a decrease in the sodium-potassium pump activity and by an increase in the permeability to cations, presumably due to alterations in the thiol-disulphide balance of the membrane proteins (Epstein, 1971). The GSH status may be involved in amino acid transport. It has been shown that amino acid (and sugar) transport is irreversibly inhibited by the thiol oxidant diamide, probably via effect on protein kinase activity (Pillion *et al.*, 1976). Orlowski and Meister proposed a different link between GSH metabolism and amino acid transport, via the γ-glutamyl moiety, in the form of a series of six enzyme catalysed reactions (Meister *et al.*, 1983). These six reactions constitute the γ-glutamyl cycle, which accounts for the

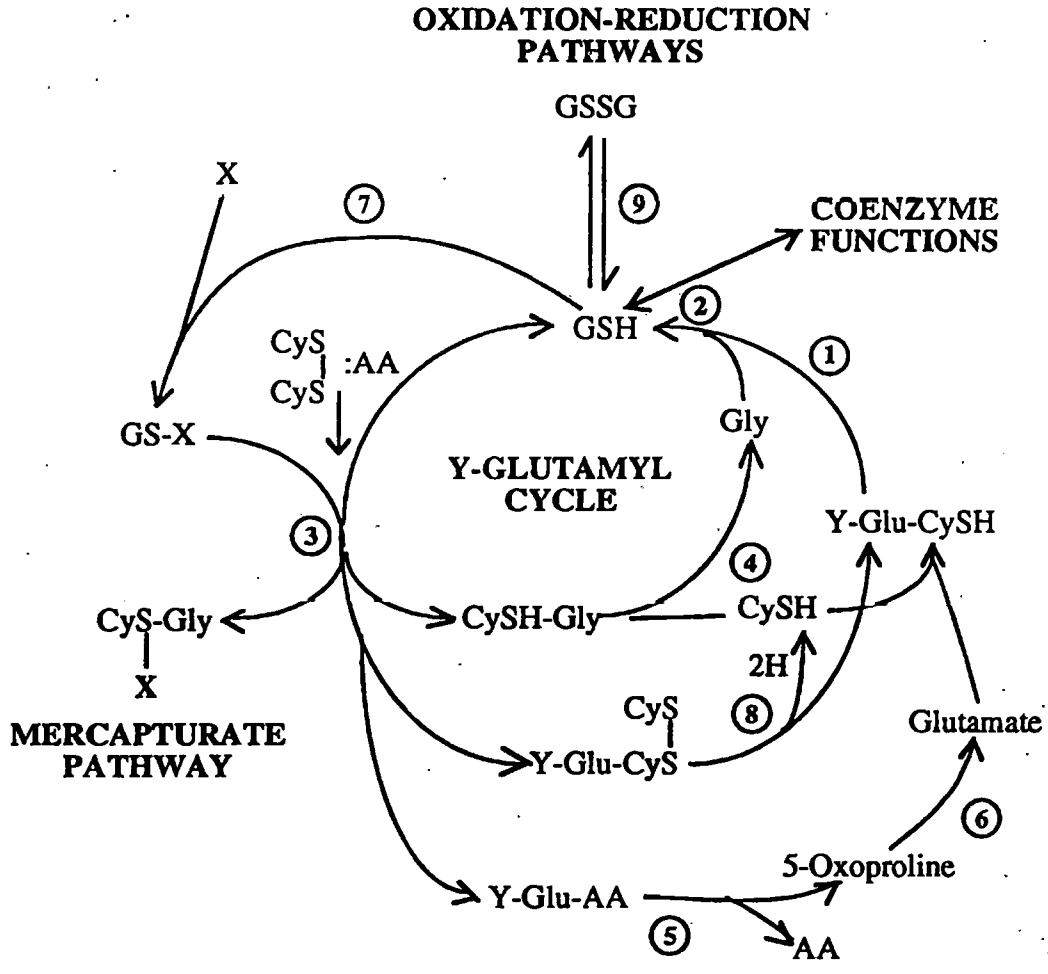
synthesis and degradation of GSH. In addition to the role of GSH in membranes, it also provides the reducing power for the conversion of ribonucleotides to deoxyribonucleotides and for a variety of thiol-disulphide interconversions. GSH is important, therefore, in the synthesis and repair of DNA and for the folding of newly synthesised proteins.

6.2.3 The metabolism of GSH

Figure 6.2 shows some of the biochemical transformations of GSH, which is synthesised in two steps from glutamate, cysteine and glycine.

Figure 6.2

Pathways of GSH metabolism

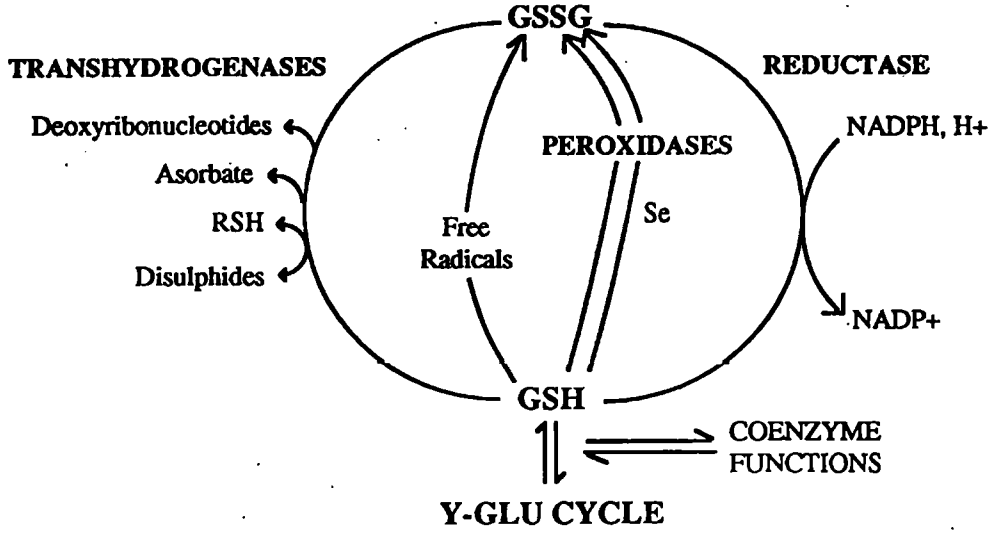


Pathways of glutathione (GSH) metabolism. 1, γ-glutamylcysteine synthetase; 2. glutathione synthetase; 3. γ-glutamyl transpeptidase; 4. cysteine glycine hydrolases; 5. γ-glyamyl cyclotransferase; 6. %-oxoprolinase; 7. glutathione S -transferase; 8. transport and reduction of γ-Glu-(CyS)2; 9. oxidation reduction pathways see figure 6.3.

Metabolic utilisation of GSH follows several pathways including reactions catalysed by the glutathione *S* -transferases (mercapturate pathway). GSH is a substrate of the glutathione peroxidases which destroy hydrogen peroxide and organic peroxides. The glutathione disulphide (GSSG) formed is reduced to GSH in a NADPH-mediated reaction. The utilisation of glutathione is initiated extracellularly by the actions of γ-transpeptidase and dipeptidase. These enzymes are bound to the outside of the cell membrane and act on GSH, GSSG and glutathione *S* - conjugates. GSH is exported by the liver to the blood plasma and to the bile. Plasma GSH is used by many tissues, for example, the kidney, lung and brain. GSH itself is not significantly transported into the cells in these tissues but is broken down by membrane bound γ-glutamyl transpeptidase and dipeptidase. The products of breakdown are transported and utilised for GSH synthesis. This is an important pathway of GSH metabolism.

Figure 6.3

Oxidation reduction pathways



6.2.4 Sensitisation of tumours to chemotherapy and radiation by BSO

As discussed in Section 1.3.3, many tumour cell lines resistant to alklating agents, for example melphalan, have increased levels of GSH. After the development of the amino acid sulfoximine inhibitors of γ-glutamylcysteinc synthetase, it was suggested that treatment with these agents may make tumour cells more sensitive to chemotherapy (Meister *et al.*, 1979). The potential usefulness of DL-buthionine S,R-sulphoximine (BSO) in the sensitisation of cells to radiation was first demonstrated in studies on several human lymphoid cell lines (Meister *et al.*, 1983; Dethmers *et al.*, 1981). Cells that had 4 to 5% of the control levels of GSH were found to be more sensitive than the controls to the effects of gamma-irradiation treatment of mice bearing B-16 melanomas (Meister *et al.*, 1986). It was also found that treatment of resistant leukaemias with BSO led to sensitisation of the tumours to phenylalanine mustard (Suzuaka *et al.*, 1983). Studies on the relationship between glutathione levels and the expression of primary drug resistance and cross resistance in human ovarian cancer cell lines and *in vivo* studies in which an i.p. model of human ovarian cancer was developed in nude mice lead to a clinical trial of BSO which is currently still in progress (Hamilton *et al.*, 1990).

6.2.5 The role of glutathione *S* -transferases in anticancer drug resistance

Section 1.2.3, introduced the fact that some cell lines selected for resistance to alkylating agents have increased activity of one or more GST isoenzymes. The data in this field are ambiguous and sometimes conflicting. The following points concerning GST and drug resistance are well established: (a) tumours express high levels of GST, especially GSTα, although the isoenzyme components vary quite considerably between tissues and the isoenzymes are inducible; (b) nitrogen mustards are good substrates for the GSTα family of isoenzymes and are frequently overexpressed in cells with acquired resistance to these drugs; (c) most drugs of the MDR phenotype have not been shown to be GST substrates and although GSTπ is

frequently overexpressed in multidrug resistant cells, most indications are that this is an accompaniment to, rather than a cause of, the resistant phenotype; (d) transfection of GST complementary DNAs (cDNAs) has produced some lines with increased resistance to alkylating agents (Tew, 1994) In summary, GSTs are important determinants of drug response for some, but not all, anticancer drugs. Most studies have overlooked the potential importance of other enzymes involved in the maintenance of cellular GSH homeostasis, and this has complicated data interpretation.

6.3 XR9173

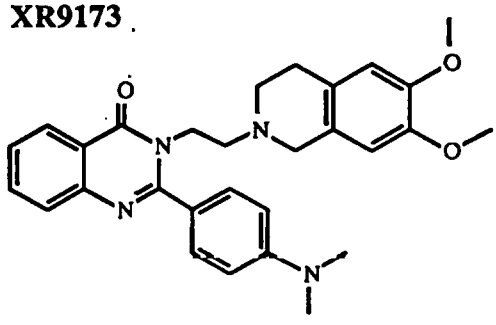
The data presented in Chapter 5 indicates that XR9173 is an efficient modifier of both P-glycoprotein- and MRP-mediated MDR. Figure 6.4 shows that XR9173 has a tetrahydroisoquinoline group within its structure. We demonstrated in Chapter 4 that the presence of this group within a molecule tends to increase its activity as a modifier of P-glycoprotein mediated MDR, possibly by increasing the positive charge of the molecule at physiological pH. We do not have a simple explanation for the activity of XR9173 as a modifier of MRP-mediated MDR. As MRP is thought to be identical to the GSH conjugate transporter (Jedlitschky *et al.*, 1994) and several groups have reported ATP-dependent transport of glutathione *S*-conjugates it is possible that XR9173 interacts with this transport system. In order to test this hypothesis we decided to examine; (a) the effect of XR9173 on the accumulation and efflux of the fluorescent, anionic dye calcein and (b) the effect of XR9173 on intracellular and released GSH levels. We know that MRP-overexpressing cells accumulate less daunorubicin and colchicine than there related parental cell lines and that XR9173 restores this accumulation deficit to levels approaching that of the parental, drug sensitive cells (Section 5.3.5). It may, therefore, be possible that XR9173 interacts with the mechanism(s) involved in the transport of cationic drugs which may well be distinct from the GSH conjugate transporter. Here we have used photoaffinity labelling studies and confocal imaging

of intracellular fluorescent dye and drug distribution to try to establish the mechanism of action of XR9173.

Figure 6.4

The structure of XR9173

XR9173



6.3 Materials and Methods

6.3.1 Cell lines

For studies involving MRP we used the human large cell lung cancer cell line, L23/P and the adenocarcinoma line MOR/P together with their respective MRP-overexpressing variants L23/R and MOR/0.4R. For studies involving P-glycoprotein we used the human T-lymphoblastoid cell line CEM together with its P-glycoprotein overexpressing variant, CEM/VLB. Details of the derivation, maintainance and culture conditions of these cell lines are described in Section 2.1.

6.3.2 Measuring drug accumulation and efflux using flow cytometry

(i) Drug accumulation

Resistance modifier (test compound) at varying concentrations and cyclosporin A 2μM were added to cells inoculated at a concentration of $2x10^5$/ml into Falcon tubes in a volume of 5ml. After 30 min fluorescent drug or dye was added to the cell suspension.. Samples were taken and drug accumulation determined by measuring intracellular fluorescence using a flow cytometer as described in Section 2.5.2.i.

(ii) Drug efflux

Cells were loaded with calcein AM (calcein AM is transformed into the highly fluorescent calcein upon cleavage of ester bonds by intracellular esterases) in the presence of probenecid (5mM) for 30 min (probenecid, an organic anion transport inhibitor, has been shown to increase calcein accumulation in L23/R cells (Versantvoort *et al.*, 1995b)). Calcein efflux was measured as described in Section 2.5.2.ii.

6.3.3 Confocal microscopy

The effect of XR9173 (5μM) on the distribution of 10μM doxorubicin and 250nM calcein in L23/P and L23/R cells was determined as described in Section 2.8.2.ii.

6.3.4 Glutathione (GSH) Assays

(i) Intracellular GSH levels

The effect of 5μM XR9173 on intracellular GSH levels in drug sensitive L23/P and MOR/P cells together with their respective MRP-overexpressing variants L23/R and MOR/0.4R was determined as described in Section 2.9.1.

(ii) Cellular GSH release

The effect of 5μM XR9173 on GSH release from L23/P and L23/R cells was determined as described in Section 2.9.2.

6.3.5 Photoaffinity Labelling

The effect of XR9173 on the ability of [^{3}H]azidopine to photolabel P-glycoprotein in membrane protein prepared from drug sensitive CEM and P-glycoprotein overexpressing CEM/VLB cells was determined as described in Section 2.6.2.

6.3.6 Calcein-AM

Calcein acetoxy-methyl ester (calcein AM) is a lipophilic, non-fluorescent compound, which rapidly permeates the plasma membrane of cells and upon cleavage of the ester bonds by intracellular esterases is transformed into the highly fluorescent calcein. Calcein, an organic anion, is thought to be a substrate or MRP (Feller *et al.*, 1995).

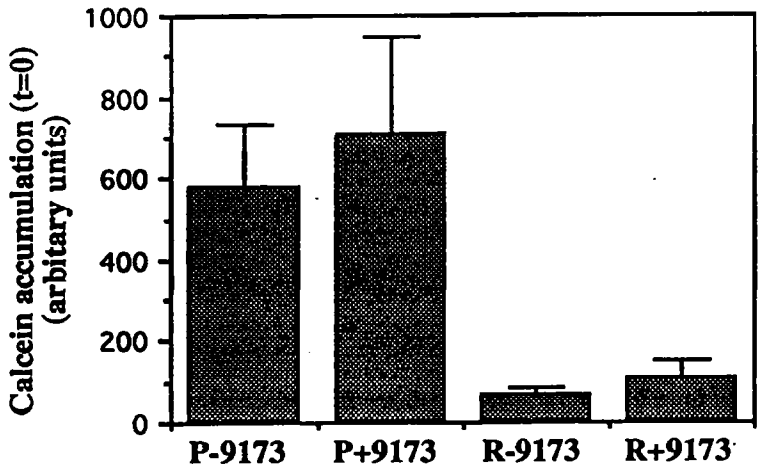
6.4 Results

6.4.1 Effect of XR9173 on the accumulation of calcein in L23/P and L23/R cells

The calcein accumulation deficit in L23/R is clearly demonstrated in figure 6.5. At t=0, the first sample taken immediately after the addition of calcein AM to the cell suspension, calcein fluorescence in L23/P was 6-fold greater than in L23/R. The addition of XR9173 to the cell suspension only modestly effected calcein fluorescence in both L23/P and L23/R.

Figure 6.5

Effect of XR9173, 5μM on Calcein accumulation in L23/P and L23/R cells at time, t=0



Values are means of at least 3 independent experiment. Error bars indicate standard error.

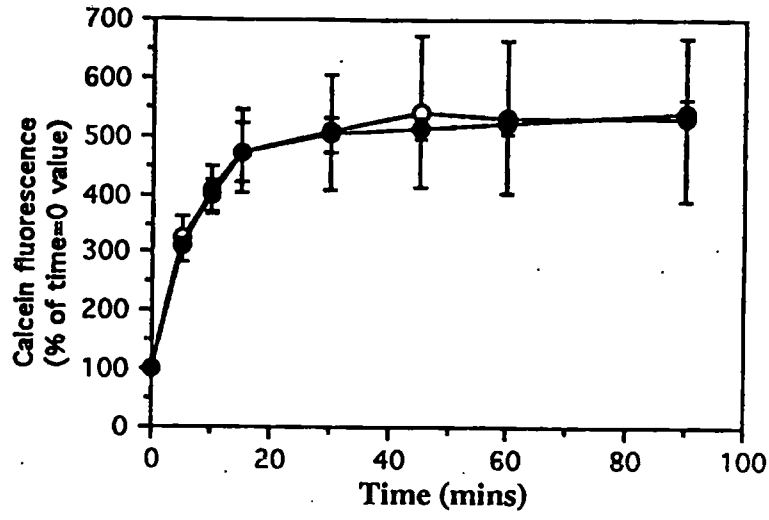
Time,t=0- the first sample taken immediately after the addition of calcein AM to the cell suspension.

Figure 6.6.a shows that XR9173 at a concentration of 5μM had no effect on Calcein accumulation over a period of 90 minutes in the drug sensitive cell line, L23/P. In contrast figure 6.6.b demonstrates that the Calcein accumulation deficit observed in the drug resistant, MRP overexpressing cell line, L23/R, was reversed on addition

of 5μM XR9173. This increase in calcein accumulation however, began to drop after 30 minutes. It should be noted, however, that these figures represent the increase in calcein accumulation in relation to the t=0 value and that this value was much smaller for L23/R than for L23/P. Even though calcein accumulation was increased by XR9173 in the resistant cell line, L23/R it was not restored to the levels observed in the drug sensitive, L23/P cell line.

Figure 6.6

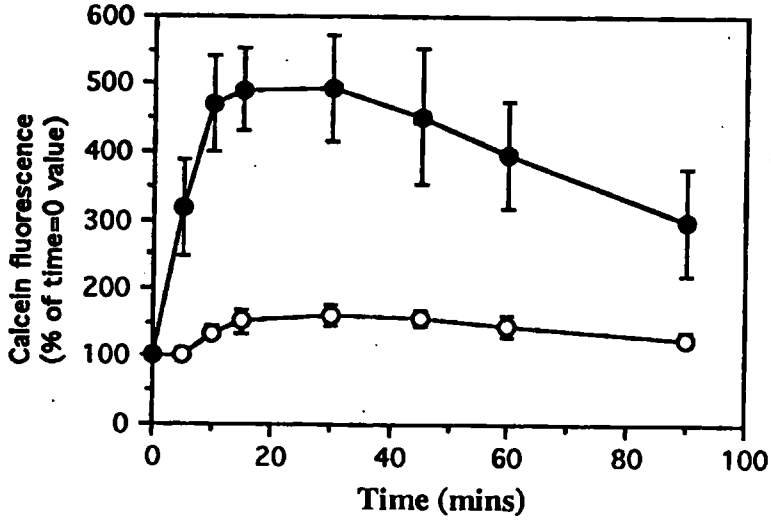
(a) Effect of XR9173 on the accumulation of calcein in L23/P cells



(●) parent,L23/P cells + XR9173 5μM, (○) parent,L23/P cells - XR9173 5μM

Values are means of at least 3 independent experiment. Error bars indicate standard error.

(b) Effect of XR9173 on the accumulation of calcein in L23/R cells



(●) resistant,L23/R cells + XR9173 5μM, (○) resistant,L23/R cells - XR9173 5μM

Values are means of at least 3 independent experiment. Error bars indicate standard error.

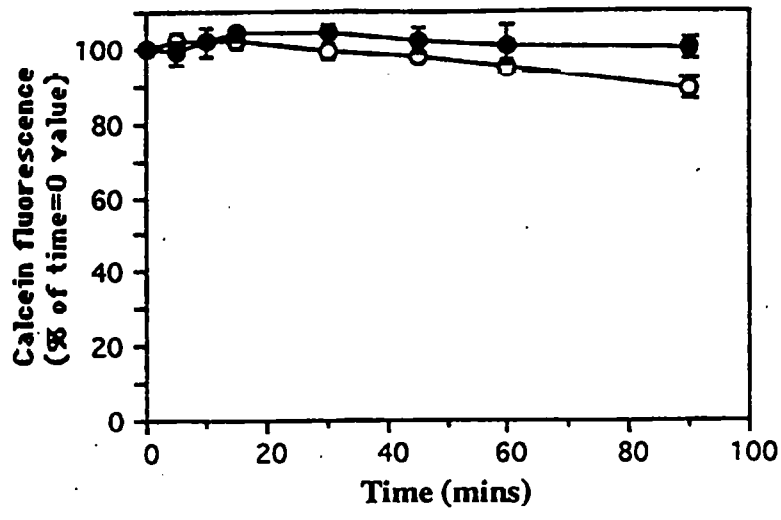
6.4.2 Effect of XR9173 on the efflux of calcein from L23/P and L23/R cells

Over a period of 30 minutes the drug sensitive cell line, L23/P retained calcein at the levels observed at t=0 (the first sample taken after the addition of calcein to the cell suspension). Calcein fluorescence then began to drop gradually over the next 60 minutes. At 90 minutes calcein fluorescence was 90% of the t=0 value (Figure 6.7.a). It appears, therefore, that there was a very small but significant (p=0.0099, Student's t test) efflux of calcein from L23/P cells over a 90 minute time period. In contrast no significant decrease in calcein fluorescence was observed in the presence of 5μM XR9173. This indicates that in L23/P the small amount of calcein efflux observed was prevented by the addition of 5μM XR9173.

In contrast to L23/P, after a period of 90 minutes calcein fluorescence decreased to 30% of the t=0 value in the drug resistant, L23/R cells (Figure 6.7.b). In the presence of 5μM XR9173, the decrease in calcein fluorescence still occured but the decline was slower. After 30 minutes calcein fluorescence was at 50% and 70% of the t=0 value in the absence and presence of XR9173 respectively. After 90 minutes, in the presence of XR9173, calcein fluorescence was at 40% of the t=0 value (compared to 30% in the absence of XR9173). These data indicate that calcein is effluxed from L23/R cells and that XR9173 partially inhibits this efflux.

Figure 6.7

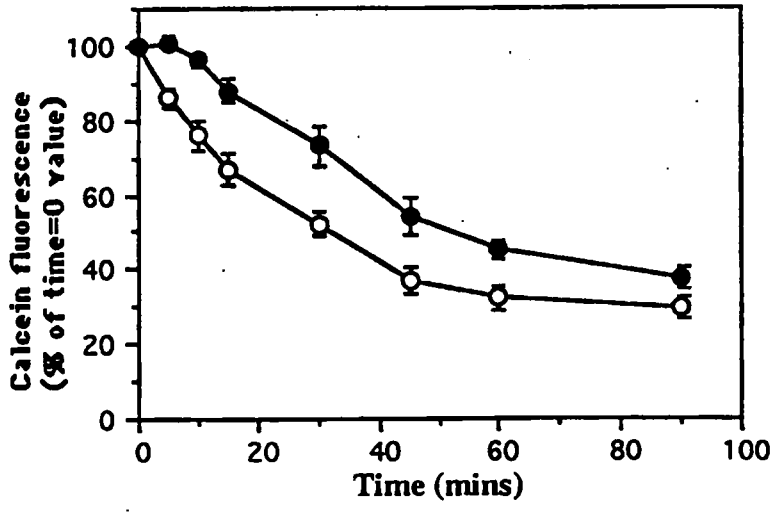
(a) Effect of XR9173 on the efflux of calcein from L23/P cells



(●) resistant,L23/R cells + XR9173 5μM, (○) resistant,L23/R cells - XR9173 5μM

Values are means (standard error)of at least 3 independent experiment.

(b) Effect of XR9173 on the efflux of calcein from L23/R cells



(●) resistant,L23/R cells + XR9173 5μM, (○) resistant,L23/R cells - XR9173 5μM

Values are means (standard error)of at least 3 independent experiment.

6.4.3 Effect of XR9173 on the intracellular distribution of calcein in L23/P and L23/R

Figure 6.8 a and b shows L23/P, drug sensitive, cells after 30 minutes exposure to 250nM calcein in the absence (Figure 6.8.a) or presence (Figure 6.8.b) of 5μM XR9173. calcein fluorescence is so intense it is difficult to establish any pattern of distribution. The black spots on the cells are areas of pixel saturation due to intense calcein fluorescence.

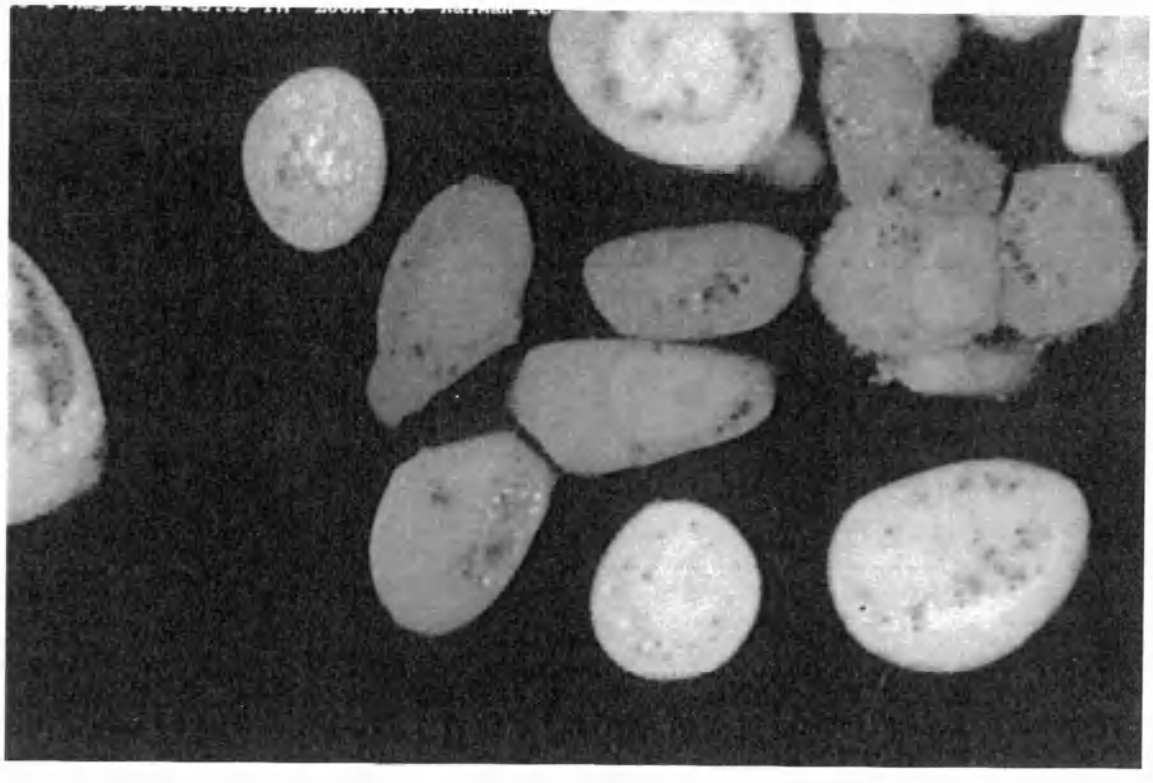
Figure 6.8 c and d shows L23/R, drug resistant, cells after 30 minutes exposure to 250nM calcein in the absence (Figure 6.8.c) or presence (Figure 6.8.d) of 5μM XR9173. Without XR9173 no intracellular calcein fluorescence is observed. On addition of 5μM 9173, intracellular calcein fluorescence is partially restored.

Confocal settings:

For the experiment shown in figure 6.8: 60X objective; Confocal aperture 5/15; Gain 630; Zoom 1.0; Neutral density 3, Kalman 10.

Figure 6.8

(a)Intracellular distribution of calcein in L23/P cells



(b) Intracellular distribution of calcein in L23/P cells in the presence of XR9173 (5μM)

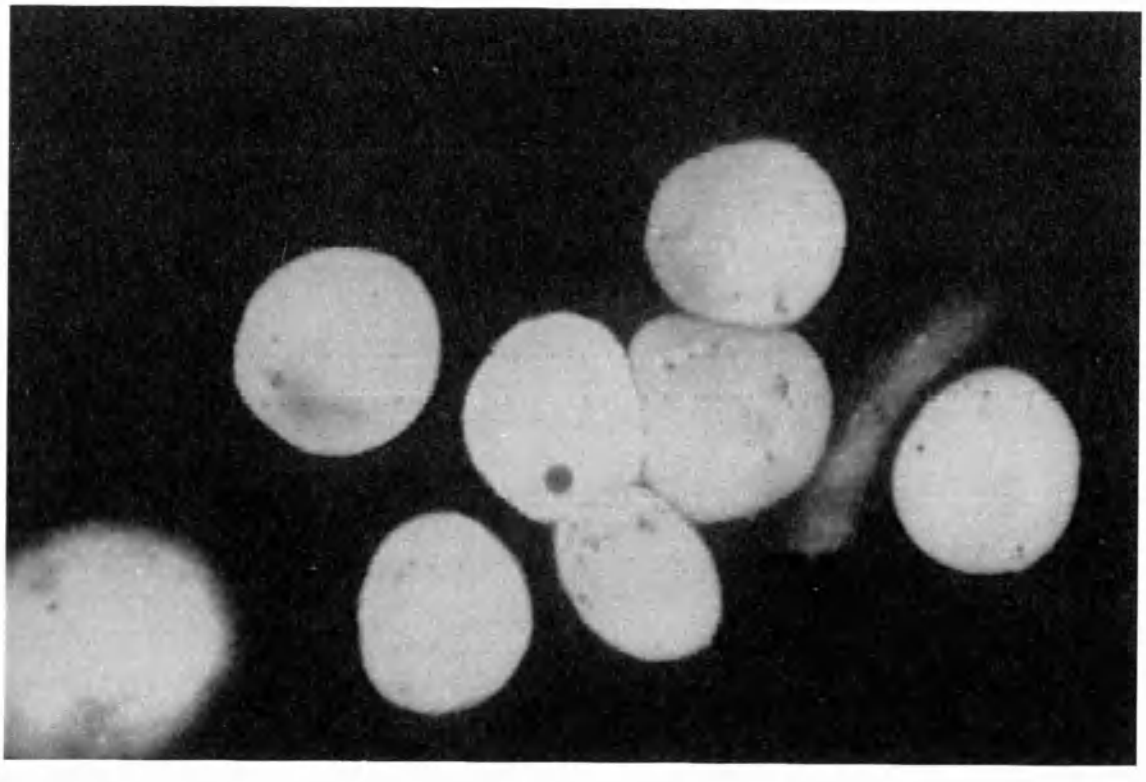
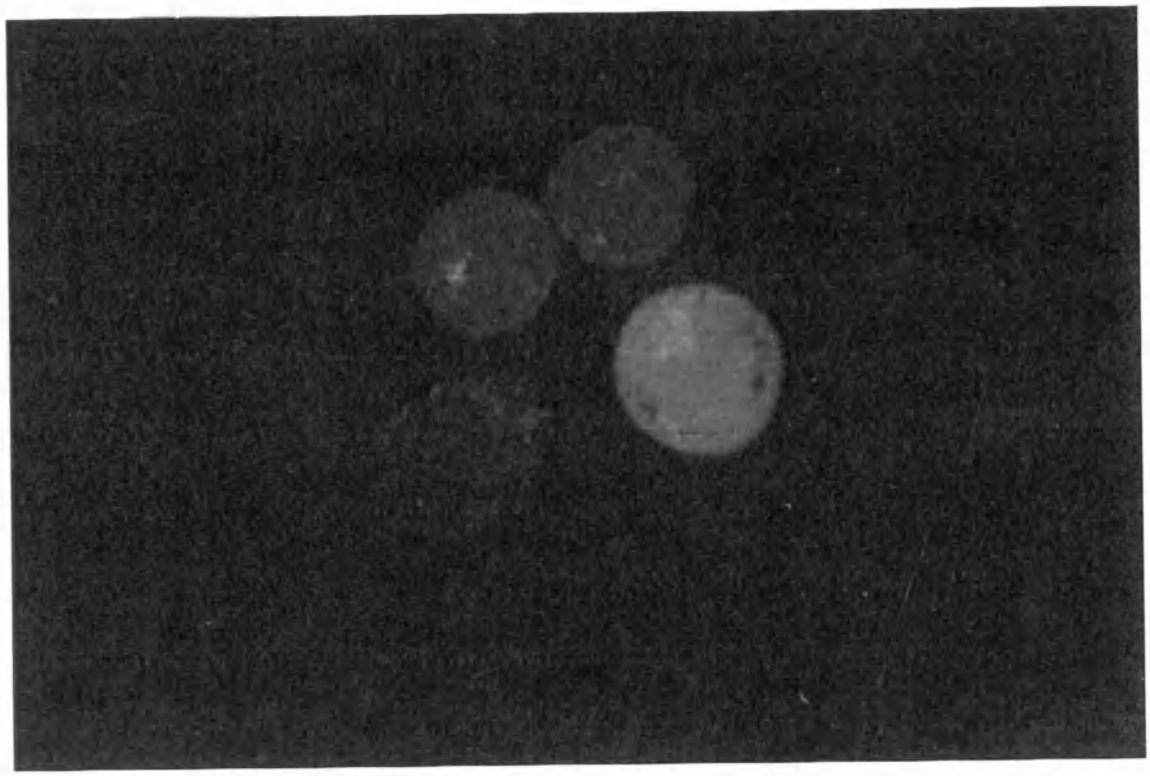


Figure 6.8

(c) Intracellular distribution of calcein in L23/R cells



(d) Intracellular distribution of calcein in L23/R cells in the presence of XR9173 (5μM)

6.4.4 Effect of XR9173 on the intracellular distribution of doxorubicin in L23/P and L23/R cells

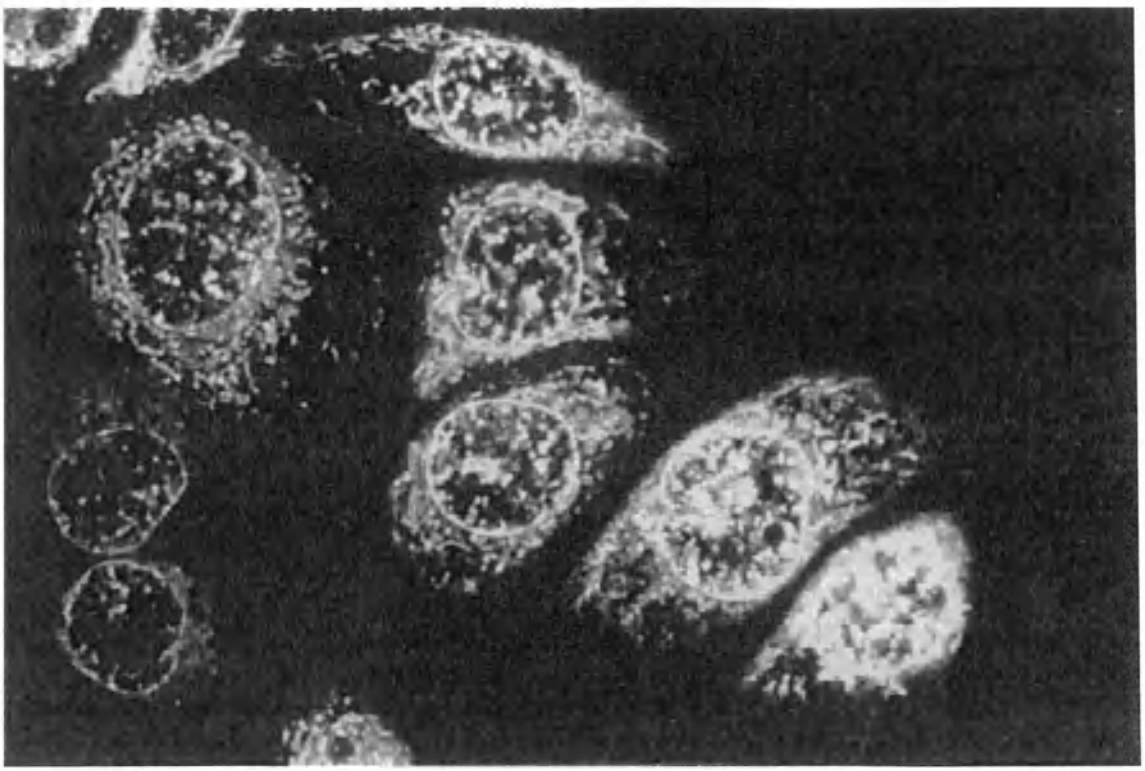
The subcellular distribution of doxorubicin inside the drug sensitive, L23/P cells and the drug resistant, L23/R cells following 2h exposures to an external drug concentration of 10μM doxorubicin was investigated under the confocal microscope exploiting the natural fluorescence of the anthracycline to visualise the drug. In the L23/P cells intense fluorescence was observed within the nucleus, at the nuclear-cytoplasmic boundary and extensively throughout the cytoplasm (Figure 6.9.a.). In the L23/R, resistant cell line, the extent of overall fluorescence was much less than in the L23/P cells. There was very little detectable fluorescence in the nucleus or the nuclear-cytoplasmic boundary. It appears that cytoplasmic fluorescence is localised into groups of vesicles lying in a distinct area around the nucleus (Figure 6.9.c.). In order to examine the effect of XR9173 on the distribution of doxorubicin in L23/P and L23/R cells, the cells were exposed to 5μM XR9173 for 30 minutes before and during the exposure of the cells to doxorubicin. In the parental cell line, XR9173 (5μM) had very little visible effect on the distribution of doxorubicin within the cell. The main areas of fluorescence are still the nucleus and the nuclear-cytoplasmic boundary. Fluorescence within the nucleus, however, appeared to be diminished compared to the parental cell line in the absence of XR9173 (5μM). The effect of XR9173 on the L23/R cell line was much more profound (Figure 6.9.d.). Fluorescence within the nucleus and the nuclear-cytoplasmic boundary were restored and even cytoplasmic fluorescence could be observed.

Confocal settings:

For the experiment shown in figure 6.9: 60X objective; Confocal aperture 5/15; Gain 800; Zoom 1.0; Neutral density 1, Kalman 10

Figure 6.9

(a) Intracellular distribution of doxorubicin in L23/P cells



(b) Intracellular distribution of doxorubicin in L23/P cells in the presence of XR9173 (5μM)

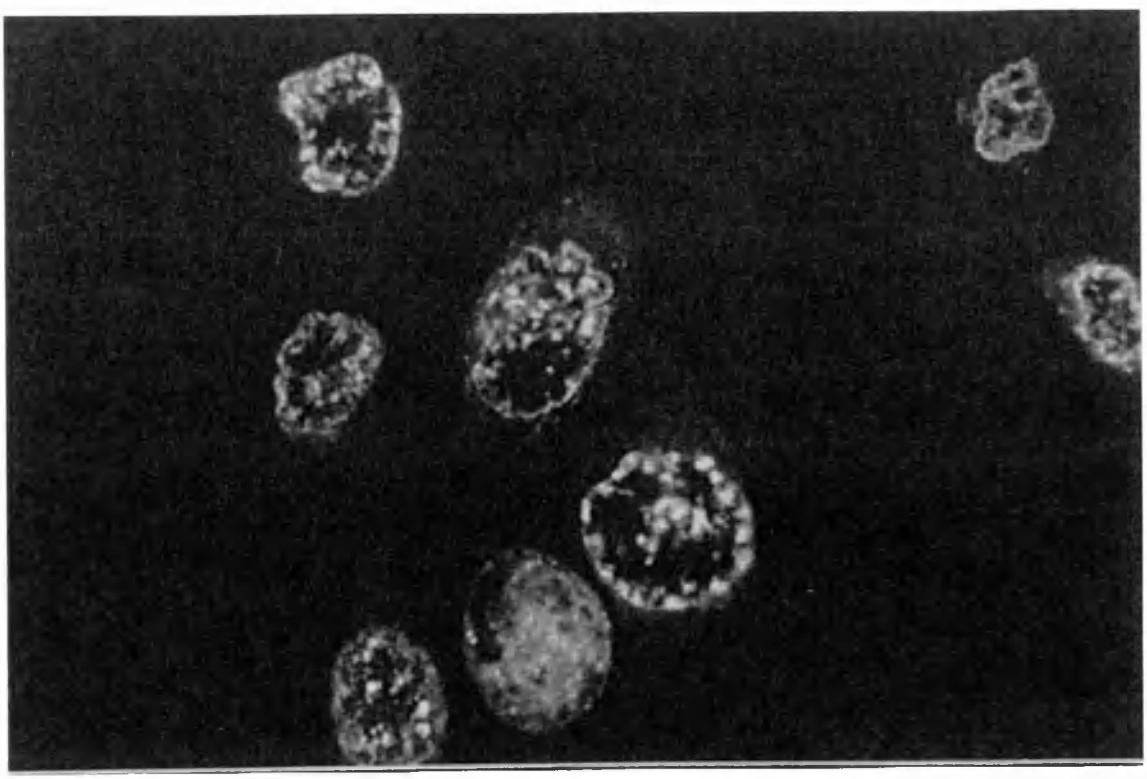
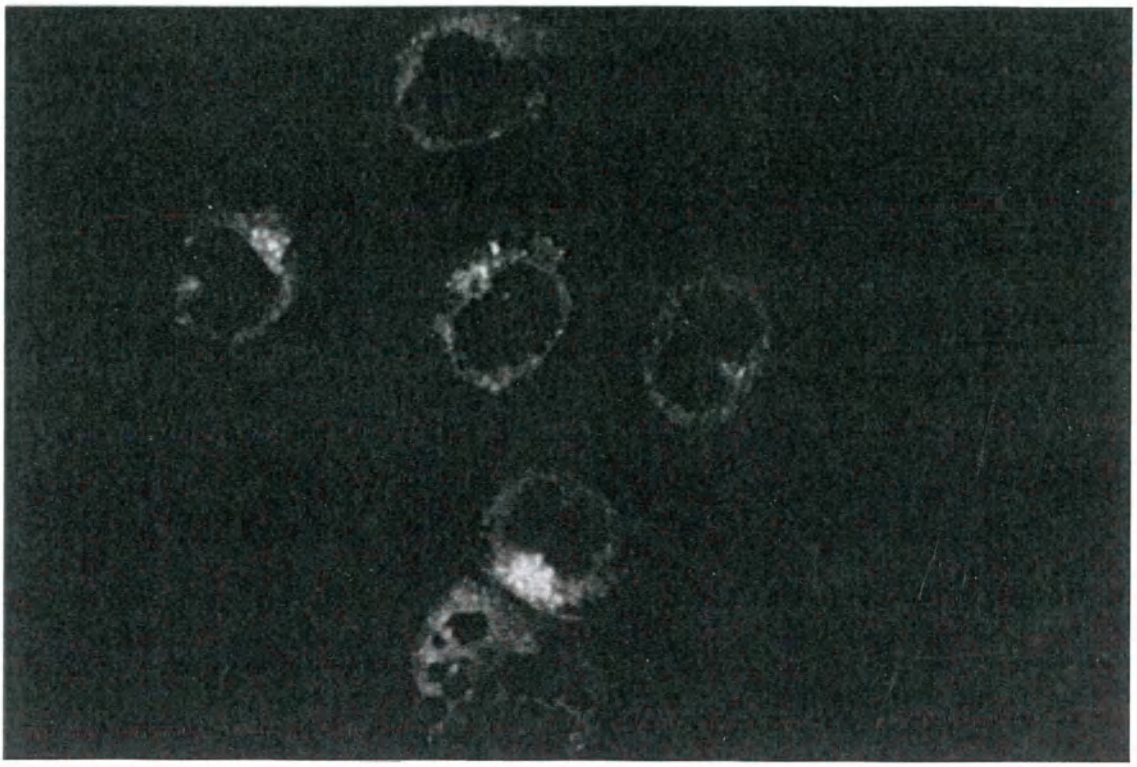
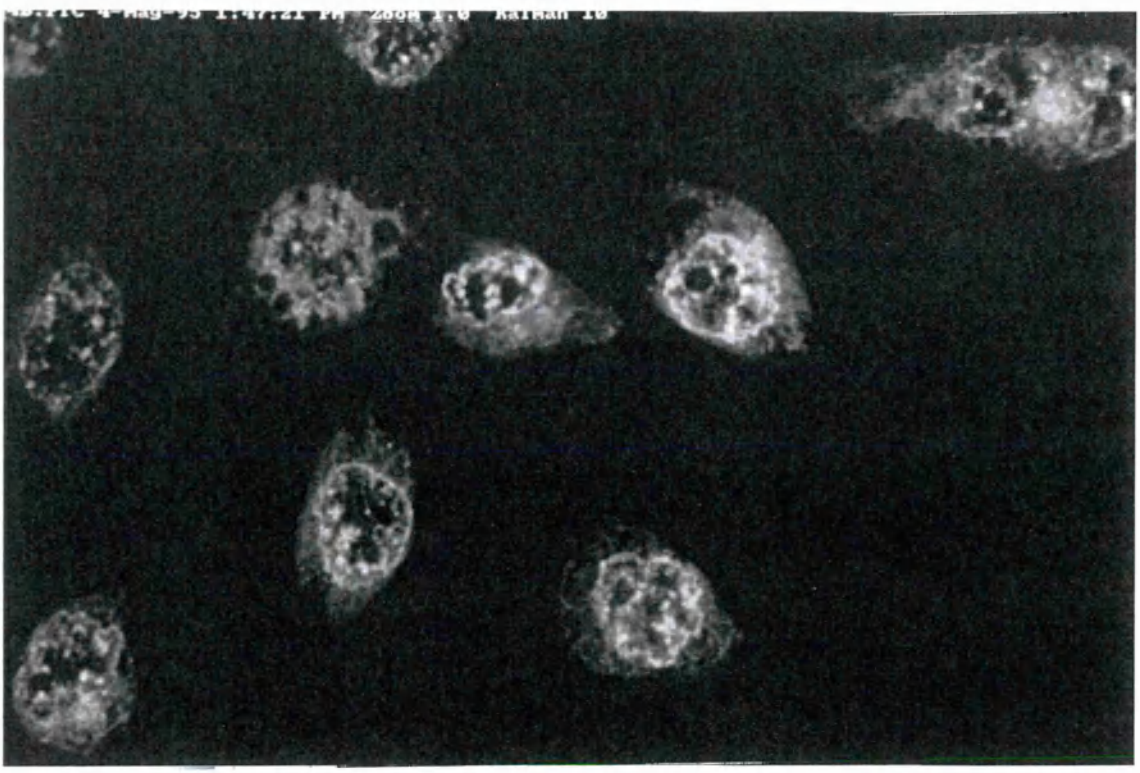


Figure 6.9

(c) Intracellular distribution of doxorubicin in L23/R cells



(d) Intracellular distribution of doxorubicin in L23/R cells in the presence of XR9173 (5μM)

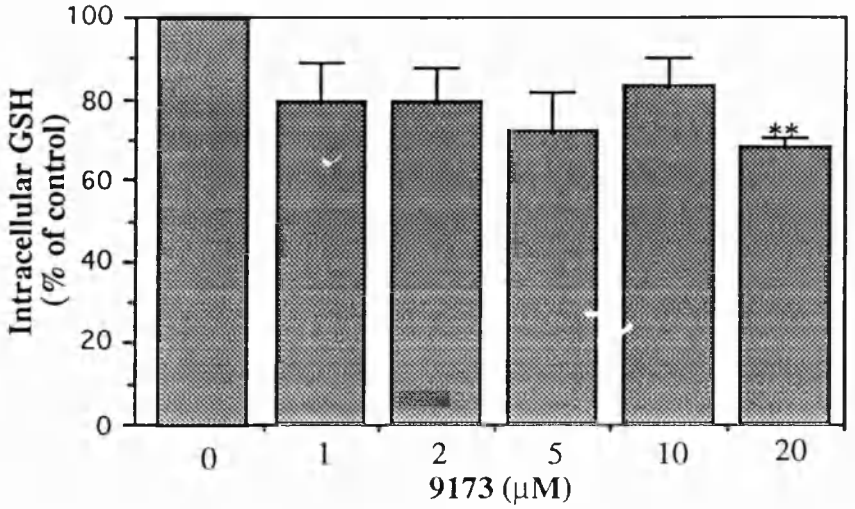


6.4.5 Effect of XR9173 on intracellular GSH levels

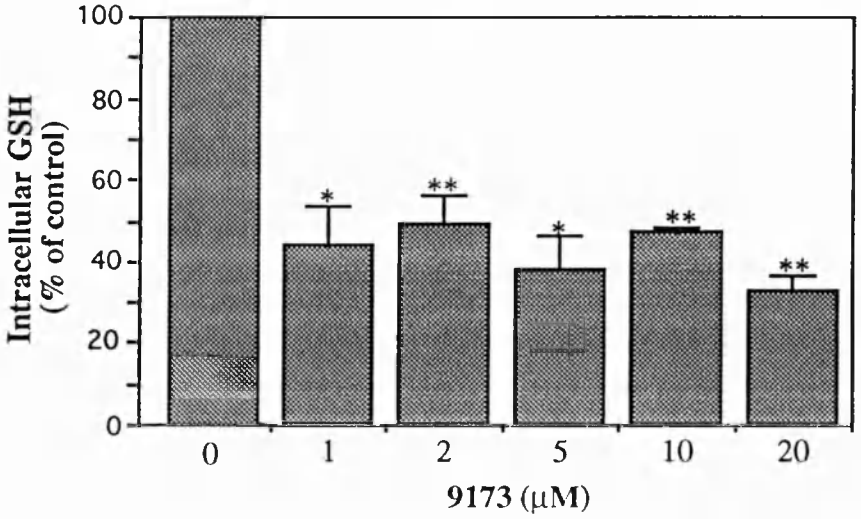
For studies involving GSH levels we used the large cell lung cancer cell line, L23/P and the adenocarcinoma cell line MOR/P together with their MRP overexpressing, resistant sublines L23/R and MOR/0.4R. In the parental MOR/P cell line, intracellular GSH levels were reduced to between 70% and 80% of the control values by XR9173 at a dose of between 1 and 20μM (Figure 6.10.a and 6.11.a). This decrease was only significant at 20μM (Student's t test). Interestingly, this decrease in GSH was not dependent on the dose of XR9173 used. In the parental L23/P cell line, 2 independent experiments showed that GSH levels were reduced to between 60% and 80% of the control values by XR9173 (1 to 20μM). Again this decrease does not appear to be dose dependent. The results were also similar for both resistant, MRP overexpressing, cell lines (Figures 6.10.b and 6.11.b). Doses of XR9173 between 1 and 20μM caused a decrease in intracellular GSH levels to between 30% and 50% of the control values (intracellular GSH in the absence of XR9173). Again this decrease in GSH levels was not dependent on the dose of XR9173 used.

Figure 6.10

(a) Effect of XR9173 on intracellular GSH levels in MOR/P



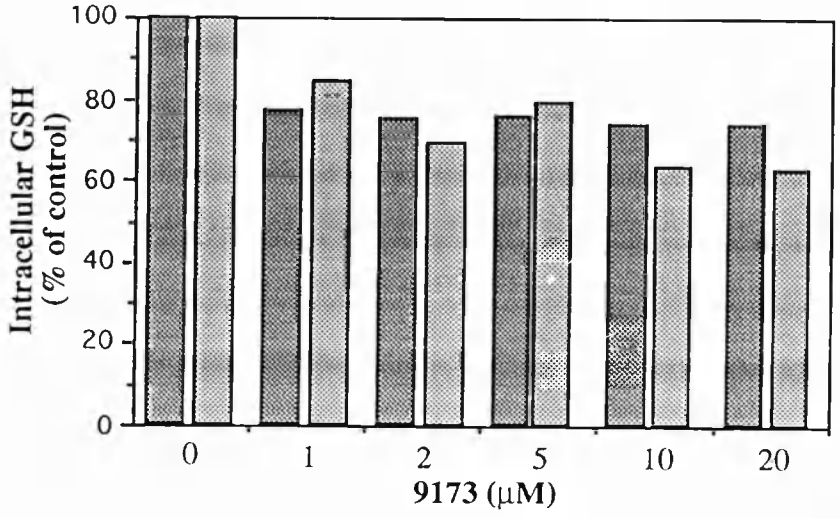
(b) Effect of XR9173 on intracellular GSH levels in MOR/0.4R



Values are means (standard error) of at least 3 independent experiment. *p<0.05 (significantly different from 100%). **p<0.01 (highly significantly different from 100%), Student's t test.

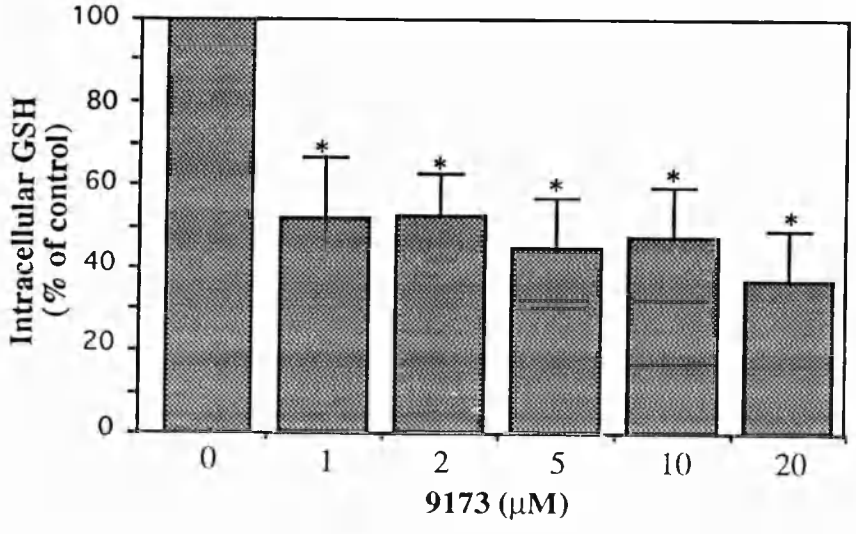
Figure 6.11

(a) Effect of XR9173 on intracellular GSH levels in L23/P



Graph shows the results of 2 independent experiment. Experiment 1 (▨), experiment 2 (□).

(b) Effect of XR9173 on intracellular GSH levels in L23/R



Values are means (standard error) of at least 3 independent experiment. *p<0.05 (significantly different from 100%). **p<0.01 (highly significantly different from 100%), Student's t test.

6.4.6 Effect of XR9173 on cellular GSH release

In order to account for the apparent decrease in intracellular GSH after exposure of MOR/P, MOR/0.4R, L23/P and L23/R cells to XR9173 we decided to examine the effect of the compound on GSH release. To establish the effect of XR9173 on cellular GSH release we measured the difference in extra cellular GSH before and after one hours exposure of L23/P and L23/R cells to XR9173. Table 6.1. shows the results obtained. In both sensitive and drug resistant cells, XR9173 appears to decrease GSH release after 60 minutes. This decrease in GSH release is not, however , statistically significant ($p=0.09$, Student's t test) in L23/P cells. In L23/R cells, however, the decrease in release does not reach significance ($p=0.03$, Student's t test).

Table 6.1

Effect of XR9173 on cellular GSH release

Cell line + modifier	GSH release (nmol/10^6 cells) after 60 min
L23/P control	5.0 ± 1.1
L23/P + 9173 (5μM)	3.4 ± 0.3
L23/R control	4.9 ± 1.4
L23/R+ 9173 (5μM)	2.9 ± 0.4*

Values are means ± standard error of at least 3 independent experiment.

*$p<0.05$ (significantly different from L23/R control value), Student's t test.

6.4.7 Effect of XR9173 on the ability of photo labelled [3H]azidopine to bind to P-glycoprotein in membranes prepared from CEM/VLB cells

The gel photograph shown in Figure 6.12 shows the effect of XR9173 on the ability of [^{3}H]azidopine to photolabel P-glycoprotein in membranes prepared from CEM/VLB cells. Lane 1 shows no azidopine binding and represents the parental CEM cell line. Lane 2 is a control and represents CEM/VLB membranes after exposure to [^{3}H]azidopine but without exposure to UV light. As [^{3}H]azidopine requires photoactivation no binding occurs. Lane 3 shows the presence of a band at a molecular weight of 170kDa representing [^{3}H]azidopine binding to P-glycoprotein. The [^{3}H]azidopine binding to P-glycoprotein is inhibited by 5μM cyclosporin A (lane 4). Lanes 5 to 9 represent the effect of XR9173 at concentrations of 0.5 to 10 μM on the ability of [^{3}H]azidopine to label P-glycoprotein. XR9173 inhibits this labelling in a dose-dependent manner.

6.5 Discussion

Calcein-AM has been recently described as an efficient functional probe for P-glycoprotein activity (Homolya *et al.*, 1993; Hollo *et al.*, 1994). The compound gives rise to fluorescent calcein following cleavage by cellular esterases. Calcein-AM has been shown to produce a large differential fluorescence between parental and P-glycoprotein overexpressing MDR cells. (Homolya *et al.*, 1993; Hollo *et al*,. 1994). In addition, it has been suggested that P-glycoprotein transports calcein-AM directly from the cell plasma membrane compartment as calcein-AM and not calcein, stimulates the vanadate-sensitive ATPase activity in isolated cell membranes of P-glycoprotein overexpressing insect cells (Homolya *et al.*, 1993). The results presented here show that, like P-glycoprotein mediated MDR cells, the MRP overexpressing cell line, L23/R also has a calcein accumulation deficit compared to the parental L23/P cell line. Our test MRP modifier, XR9173 was able partially to restore this accumulation deficit. It should be noted that the differential in accumulation between L23/P and L23/R for calcein is much larger than for daunorubicin or colchicine (see Section 5.3.5). Also XR9173 (5μM) restores the accumulation of daunorubicin and colchicine to 71% and 86% respectively of the parental levels after one hour. In comparison XR9173 (5μM) is only able to restore the calcein deficit in L23/R cells to >20% of the parental value. Versantvoort *et al.* (1995b) also demonstrated that L23/R cells have a much larger (and more rapid) accumulation deficit for calcein than for daunorubicin and rhodamine 123. The group also observed that unlike daunorubicin and rhodamine 123, calcein accumulation is not restored in L23/R cells following depletion of cellular GSH with BSO. It appears that the transport of the anionic dye, calcein and the cationic drugs, daunorubicin and colchicine is not equivalent and that differences in the mechanism(s) of transport exist. As discussed earlier in this chapter, several groups have reported ATP-dependent transport of glutathione *S*-conjugates (Jedlitschky *et al.*, 1994; Muller *et al.*, 1994). Leier *et al.* (1994a) demonstrated that photoaffinity labelling of mastocytoma cell membranes with LTC_4, the GSH conjugated

leukotriene C_4, specifically labels a 190-kDa plasma membrane protein. This is the molecular weight of MRP. The group have also demonstrated that MRP mediates the ATP-dependent transport of LTC_4 and structurally related conjugates in membrane vesicles from HeLa cells transfected with an MRP expression vector (Leier *et al.*, 1994b). These findings have lead to the recent suggestion that MRP is identical to the glutathione conjugate transporter present in hepatocytes, macrophages and other normal cell types (Jedlitschky *et al.*, 1994). It seems likely considering the data presented here that calcein may be a model substrate for MRP partially inactivating the MRP pump by competing for the same efflux mechanism as GSH. Versantvoort *et al.* (1995b) postulate that efflux of daunorubicin and rhodamine 123 occurs via a mechanism for which MRP action is necessary but not sufficient. They found that several agents were capable of modulating the transport of daunorubicin and rhodamine 123 such as, genistein, verapamil, BSO, probenecid, orthovanadate and oubain in L23/R cells. Only probenecid, however, restored calcein accumulation. The data presented here indicate that, like probenecid, XR9173 is also able efficiently to inhibit the transport of daunorubicin and partially restore the calcein accumulation deficit in L23/R cells. It may, therefore, have a dual function being able to inhibit (partially) the organic anion transporter and also inhibit the, as yet undefined, mechanism responsible for causing a decreased intracellular accumulation of cationic cytotoxic drugs. Interestingly, XR9173 is able to modulate drug transport in both MRP overexpressing and P-glycoprotein overexpressing MDR cell lines. The compound is also able to inhibit the labelling of P-glycoprotein by [^{3}H]azidopine indicating that the compound may be a substrate for P-glycoprotein. Like other known P-glycoprotein-mediated MDR modulators such as cyclosporin A and verapamil, it may modulate the transport of cytotoxic drugs by competitive inhibition of the P-glycoprotein drug efflux pump. Attempts to label MRP with [^{3}H]azidopine were unsuccessful and it is not known whether XR9173 is also a substrate for MRP. It may be possible that the MRP transporter may regulate a related mechanism which is resposible for daunorubicin transport

anion transporter thought to be identical to MRP. It does, however, modulate the accumulation and efflux of calcein, an anionic fluorescent dye. The results presented here provide evidence to support the theory that MRP may co-exist with another transporter which is able to transport cationic drugs across the membrane and that these transport are able in some way to regulate each others activity.

Chapter 7

Modification of phosphorylation of P-glycoprotein and of drug transport by activators and inhibitors of protein kinase C

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7.4 Discussion

7.1 Introduction

7.1.1 Protein kinase C (PKC)

Protein kinases are regulatory enzymes which catalyse the covalent attachment of phosphate groups to proteins. This phosphorylation of target proteins induces conformational changes, which result in modified cellular activities. Protein kinases are now recognised as biological levers of immense importance which regulate, in a complex fashion, steps essential for cellular differentiation and proliferation (Gescher *et al.*, 1994). In eukaryotic cells, protein kinases fall, generally, in to two general classes : those which transfer phosphate to serine or threonine residues and those which phosphorylate tyrosine. One of the best studied kinases is the serine/threonine specific protein kinase C (PKC). PKC was first discovered by Nishizuka and co-workers in 1977, who described it as a histone protein kinase activated by Ca^{2+}, phospholipids and diacylglycerol or phorbol esters. PKC is now known to consist of a single polypeptide chain of 77-83kDa, made up of a regulatory domain that interacts with enzyme cofactors and a catalytic domain which transfers ATP-derived phosphate to acceptor substrates. The regulatory domain also contains the 'pseudosubstrate', an amino acid sequence closely resembling the PKC substrate recognition sites, which blocks access of substrates to the catalytic site in a reversible manner. In its inactive state it is thought that PKC is located predominantly in the cytosol. On activation it associates tightly with cellular membranes, a process referred to as PKC 'translocation' or 'redistribution'. Sustained activation of PKC results in its own down regulation, mediated by proteolytic enzymes, a mechanism presumably designed to ameliorate the potentially disastrous consequences of unabated activation. Clarification of the role of PKC is complicated by the fact that PKC is an enzyme family with at least 11 different isoenzymes (α, $\beta1$, $\beta2$, γ, δ, ε, θ, η, λ, ζ and μ). These enzymes are differentially expressed in cells, have different requirements for activation and are regulated independently by activators such as the tumour-promoting phorbol esters (Nishizuka, 1992).

Little is known about differences in cellular function between the individual PKC isotypes, although the expression of more than one isoform in a particular cell type suggests that distinct PKCs may activate different cellular pathways and phosphorylate different substrates (Pongracz *et al.*, 1995).

7.1.2 Cellular function of PKC

As activated PKC is predominantly localised in cellular membranes, the majority of PKC target proteins are also associated with membranes. It is also possible, however, that a proteolytically generated fragment of PKC, comprising the catalytic domain but not the regulatory domain, is able to phosphorylate cytosolic proteins crucial for cell function (Gescher *et al.*, 1994). Focal adhesion contacts, discrete regions of the plasma membrane, appear to be important PKC substrates. These sites which are large bundles of actin filaments located at the cytoplasmic face of the plasma membrane, function as anchors for cytoskeletal stress fibres. They ensure a physical transmembrane connection between the cytoskeleton of neighbouring cells or between cytoskeleton and extracellular matrix (Jaken *et al.*, 1992). Phosphorylation of proteins which are associated with focal contacts is thought to be responsible for the transient morphological changes of cells which occur prior to cell division. The myristoylated alanine rich C kinase (MARCK) protein family is one of the most extensively studied PKC substrates (Aderem *et al.*, 1992). MARCKs, which binds to both actin and calmodulin with Ca^{2+}, is capable of cycling between the plasma membrane and the cytosol in a phosphorylation-dependent manner. On phosphorylation of MARCKs by PKC there is a rapid translocation of the protein from the plasma membrane to the cytosol, where it associates with F-actin. On dephosphorylation MARCKs reassociates with the membrane. The protein is able, therefore, to provide a regulated cross bridge between F-actin and the plasma membrane. Other important PKC substrates include members of the *src* oncogene family, the epidermal growth factor receptor and the amiloride-sensitive Na^+/H^+ exchanger, a plasma membrane glycoprotein which regulates intracellular pH.

In 1990, Chambers and co-workers were the first to demonstrated that P-glycoprotein, the ATP-dependent drug efflux pump associated with multidrug resistance in cancer cells, is a substrate for PKC *in vitro*. The group also demonstrated that stimulation of P-glycoprotein phosphorylation by phorbol esters *in vivo* leads to reduced drug accumulation in vincristine-resistant KB cells (Chambers *et al.*, 1990a & b). A recent study by Ma *et al.* (1995) demonstrates that the 190kDa, multidrug resistance associated protein (MRP) is highly phosphorylated and that the phosphorylation levels can be reduced by inhibitors of PKC. This suggests that MRP may also be a substrate for PKC (Ma *et al.*, 1995). The role of PKC in MDR is very controversial and has been the subject of intense research of late. This issue is discussed in detail later in this chapter.

The role of PKC is indeed very complex. The discovery that a whole range of isoenzymes that are differentially expressed in various cells and tissues and that vary both in activation conditions and substrate specificity partially explains how a protein that at first appeared to be a single ubiquitous enzyme is able to control so many diverse functions.

7.1.3 Inhibitors of PKC

PKC inhibitors can be divided into two broad classes: Those which inhibit the activity of the catalytic domain, competing with the phosphate group, donor ATP or the substrate; and those which target the regulatory domain of the enzyme, competing with cofactors. The indole carbazole, microbial product staurosporine is the prototypical protein kinase inhibitor. It interacts with the catalytic, kinase domain of PKC with high potency. Due to the ATP-competitive nature of its inhibitory action it also impedes a number of other kinases with similar potency. Staurosporine analogues have been developed which appear to be more selective for PKC, whilst retaining high potency for enzyme inhibition. Examples are the hydroxylated

staurosporine derivative UCN-01 developed by Kyowa Hakko in Japan (Takahashi *et al.*, 1987) and the N-benzoyl derivative CGP 41251 (Meyer *et al.*, 1989) from Ciba Geigy in Switzerland. The PKC inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), also inhibits PKC at the catalytic domain and is less specific for PKC than staurosporine. The fungal product, calphostin C, is a relatively selective PKC inhibitor (Kobayashi *et al.*, 1989) which irreversibly targets the phorbol ester/diacylglycerol, regulator, binding site of PKC by light-dependent oxidation (Bruns *et al.*, 1991).

Lysophingolipids such as sphingosine are metabolites of membrane sphingolipids and are therefore natural constituents of cells. They have been shown to inhibit the regulatory domain of PKC.

A number of drugs that are currently in clinical use, such as chlorpromazine, doxorubicin and suramin, are moderately effective PKC inhibitors. One of the most effective is the anti-oestrogen, tamoxifen. Tamoxifen and certain of its metabolites inhibit PKC at the regulatory domain at clinically effective drug concentrations (O'Brian *et al.*, 1985). It has been suggested that PKC inhibition may contribute to the anti-breast cancer activity of tamoxifen.

7.1.4 Activators of PKC

Activators of PKC, such as the tumour-promoting phorbol ester TPA, cause a plethora of morphological and biochemical effects in cells (Blumberg, 1980). In some cell lines, exposure to PKA activators causes mitogenesis, yet in others it leads to inhibition of proliferation. The biochemical events which trigger the biological effects of PKC activators are only poorly understood. It is unlikely that the activation of PKC on its own would be sufficient for some of these events to occur. It seems that a cellular response to exposure to phorbol esters may result from the interplay of a variety of factors in concert with PKC activation. Studies have reported that

enhancement of PKC activity of the phorbol ester TPA can increase vinblastine transport in MDR cells (Fine *et al.*, 1988; Chambers *et al.*, 1992; Bates *et al.*, 1993). Since P-glycoprotein phosphorylation is increased by TPA treatment, these results suggest that alterations in phosphorylation can modulate drug transport. Interestingly, prolonged exposure of cells to TPA results in a down regulation of PKC levels (Nishizuka *et al.*, 1988).

7.1.5 The role of PKC and protein phosphorylation in multidrug resistance

A potential regulatory mechanisms for P-glycoprotein function is covalent modification by phosphorylation. It is now evident that phosphorylation is a universal characteristic of the P-glycoproteins expressed in MDR cells (Roy *et al.*, 1985; Chambers *et al.*, 1990b) and is a dynamic process, with rapid phosphorylation/dephosphorylation cycles (Ma *et al.*, 1991). The protein kinases involved in P-glycoprotein phosphorylation have not been completely characterised. Protein kinase C has been implicated because phorbol ester (an activator of PKC) stimulation of P-glycoprotein phosphorylation has been documented in several MDR cell lines (Hamada *et al.*, 1987; Yu *et al.*, 1991; Chambers *et al.*, 1992; Bates *et al.*, 1992), PKC phosphorylates P-glycoprotein *in vitro* (Chambers *et al.*, 1990b; Posada *et al.*, 1989), and the *in situ* and *in vitro* phosphorylation sites are very similar if not identical (Chambers *et al.*, 1990a & b and 1992). In addition to regulation by PKC, P-glycoprotein is also phosphorylated *in vitro* by cyclic AMP-dependent protein kinase (PKA) (Mellado *et al.*, 1987; Miyamoto *et al.*, 1990) and by a calcium-independent, phospholipid-dependent protein kinase (Staats *et al.*, 1990). It appears that P-glycoprotein may therefore be phosphorylated through the activation of different signal transduction pathways. The functional role of phosphorylation of P-glycoprotein has been evaluated by measurements of drug accumulation in MDR cells treated with phorbol ester and protein kinase inhibitors. Chambers *et al.* (1990a & b and 1992) demonstrated that phorbol ester-induced PKC activation and increased P-

7.1.6 Aims

We observed that, at doses where some of the Xenova compounds were effective modifiers of P-glycoprotein-mediated MDR, they did not inhibit the photolabelling of P-glycoprotein with [^{3}H]azidopine (Chapter 4). This suggested that the mechanism of action of these compounds may be one other than competitive binding to P-glycoprotein. As discussed already in this chapter, many groups have shown that P-glycoprotein is phosphorylated and that it is a substrate for PKC. We therefore decided to examine the effect of the XR compounds on the phosphorylation of P-glycoprotein. Meanwhile scientists at Xenova tested the ability of the compounds to inhibit PKC and found that none of the compounds tested were able to inhibit this enzyme. A US company called Novascreen also tested the compounds in a series of receptor binding assays. In a test for the ability of the compounds to inhibit the binding of the phorbol ester PDBu to PKC all the compounds were inactive. It is, therefore, unlikely that the Xenova compounds effect drug resistance via modifying the phosphorylation status of P-glycoprotein. At this stage we had already set up the phosphorylation assay and begun to look at the effect of known PKC inhibitors and the PKC activiator TPA on the phosphorylation of P-glycoprotein. The results of these initial studies proved interesting and so we decided to continue with this study. The aims of the work presented in this chapter have, therefore, changed. The initial idea was to determine whether the effect of the Xenova compounds on P-glycoprotein function was due to their effect on P-glycoprotein phosphorylation status. After considering the results obtained by scientists at Xenova we decided to continue to pursue the investigations into the effect of altering P-glycoprotein phosphorylation on drug transport. Our new aims were as follows:

(i) To examine the effect of inhibitors of protein kinase C on drug transport in the MDR cell line CEM/VLB and the ability of this cell line to transport cytotoxic drugs;

(ii) To establish whether any effect on drug transport in CEM/VLB caused by PKC inhibitors is due to the inhibitor binding directly to P-glycoprotein or to the inhibition of phosphorylation of P-glycoprotein by the inhibition of PKC.

7.2 Materials and Methods

7.2.1 Cell lines

We used the human T-lymphoblastoid cell line CEM and the small cell lung cancer cell line H69/P together with their P-glycoprotein overexpressing variants, CEM/VLB and H69/LX4 respectively. Details of the derivation, maintenance and culture conditions of these cell lines are described in Section 2.1.

7.2.2 Drug sensitivity testing

The cytoxicity of the PKC inhibitors was determined using the tetrazolium (MTT) reduction assay. This method is described in detail in Section 2.3.

7.2.3 Drug accumulation studies

The effect of the PKC inhibitors and TPA on the ability of CEM and CEM/VLB cells to accumulate [^{3}H]daunorubicin and [^{3}H]colchicine was determined as described in Section 2.5.1.ii

7.2.4 Phosphorylation of P-glycoprotein in membranes

In order to determine the effect of inhibitors and activators of protein kinase on the phosphorylation status of P-glycoprotein we adapted the method of Staats et al. 1990. Membranes, prepared from H69/P and H69/LX4 cells using the method outlined in Section 2.6.1 were incubated in a 25µl reaction as described in Section 2.7.1 Briefly, incubations were carried out for 5 min on ice. The reaction was stopped by the addition of 10mM EDTA. After the addition of 5µl sample buffer the samples were electrophoresed on a 7.5% polyacrylamide gel as described in Section 2.7.2.

7.2.5 Phosphorylation and immunoprecipitation of P-glycoprotein in intact cells

In order to correlate the effect of various protein kinase inhibitors on the transport of cytotoxic drugs in MDR cells and P-glycoprotein phosphorylation, we decided to examine P-glycoprotein phosphorylation in intact cells.

Cell lysates were prepared from [^{32}P]orthophosphate labelled drug sensitive, CEM and drug resistant, CEM/VLB cells as described in Section 2.7.3.ii P-glycoprotein was then immunoprecipitated using the monoclonal antibody, C219 as described in Section 2.7.3.iii

7.2.6 Photoaffinity Labelling

The effect of PKC inhibitors and TPA on the ability of [^{3}H]azidopine to photolabel P-glycoprotein in membrane protein prepared from drug sensitive CEM and P-glycoprotein overexpressing CEM/VLB cells was determined as described in Section 2.6.2.

7.2.7 Light activation of calphostin C

The more selective PKC inhibitor, calphostin C irreversibly targets the regulatory domain of PKC in a reaction involving light-dependent oxidation. Experiments to access the ability of calphostin C to modulate drug transport and to inhibit the phosphorylation of P-glycoprotein have, therefore, been carried out under light and dark conditions.

Light activation:

For accumulation assays; cell suspensions were placed in Falcon tubes and gased by blowing CO_2 over the surface of the cell suspension before sealing the tube with the cap. The appropriate dilutions of calphostin C were then added to each tube. The tubes were then exposed to a fluorescent light for 10 minutes. The cells were then

placed in 2ml Eppendorf tubes and incubated at 37°C for a further 20 mins before the addition of the radiolabelled drug.

For MTT assays; cell suspensions were placed in falcon tubes and gased by blowing CO_2 over the surface of the cell suspension before sealing the tube with the cap. The appropriate dilutions of calphostin C were then added to each tube. The tubes were then exposed to a fluorescent light for 10 minutes. The tubes were then incubated for 2 hours at 37°C in an incubator. After this time the cells were pelletted and rinsed twice with 2ml ice PBS. Finally the cells were resuspended in medium and seeded into 96 well plates. The plates were incubated for 4 days in an incubator at 37°C in an atmostphere of 8% CO_2. The assay was read as described in Section 2.3.

For photoaffinity labelling studies; various dilutions of calphostin C were added to membrane dilutions in 96 well plates. The plates were exposed to fluorescent light for 10 minutes before the addition of [^{3}H]azidopine. The experiment was then continued as described in Section 2.6.4.

For phosphorylation assays; cells were incubated in phosphate free medium for 2h in Falcon tubes. After this time the appropriate dilutions of calphostin C were added to each tube. The tubes were then exposed to a fluorescent light for 10 minutes. The cells were then placed in 2ml eppendorf tubes and incubated at 37°C for a further 20 mins before the addition of [^{32}P]orthophosphate. The assay was continued as described in Section 7.2.5.

For experiments involving non-light activated calphostin C, the above procedures were followed with the exception of light exposure. Any cells exposed to calphostin C were kept in the dark.

7.3 Results

7.3.1 Effect of protein kinase C inhibitors on the accumulation of [^{3}H]daunorubicin and [^{3}H]colchicine in CEM and CEM/VLB cells

In order to examine the effect of the protein kinase C (PKC) inhibitors calphostin C, H7 and staurosporine on the transport of cytotoxic drugs in MDR cells we decided to measure the ability of these PKC inhibitors to modulate the transport of the tritiated drugs, [^{3}H]daunorubicin and [^{3}H]colchicine in drug sensitive CEM and drug resistant CEM/VLB cells. The values in table 7.1. represent accumulation ratios. Accumulation ratio is defined by [^{3}H]drug accumulation in the presence of modifier (PKC inhibitor) divided by [^{3}H]drug accumulation in the absence of modifier (PKC inhibitor). Staurosporine at concentrations between 0.5 and 2.0μM had no significant effect on the accumulation of either drug in either the parental cell line, CEM (2.0μM result shown in figure 7.7.) or the drug resistant, P-glycoprotein overexpressing cell line, CEM/VLB (Table 7.1.). Like staurosporine, H7 at concentrations of 10 and 20μM also had no effect on drug transport in either the parent or resistant cell line (Table 7.1. and Figure 7.7.). The more selective PKC inhibitor, calphostin C irreversibly targets the regulatory domain of PKC in a reaction involving light-dependent oxidation. Experiments to access the ability of calphostin C to modulate drug transport have therefore been carried out under light and dark conditions. The data in table 7.1. demonstrates that light activated calphostin C increased the accumulation of both [^{3}H]daunorubicin and [^{3}H]colchicine in drug resistant, CEM/VLB cells to a much greater extent than non-light activated calphostin C. Non-light activated calphostin C, however, produced a modest but statistically significant increase in the accumulation of [^{3}H]daunorubicin but not [^{3}H]colchicine. Figures 7.1 and 7.2. show graphical representations of the difference in the effect of calphostin C on the accumulation of [^{3}H]daunorubicin and [^{3}H]colchicine in drug sensitive CEM cells and drug resistant CEM/VLB cells under light and dark conditions. There was no significant (Student's t test) effect of calphostin C (dark and light) on the

accumulation of [^{3}H]colchicine in the parental cell line, CEM. Light activated calphostin C (400nM) did significantly increase the accumulation of [^{3}H]daunorubicin in the drug sensitive CEM cell line ($p=0.017$, Student's t test) but had no significant effect on the accumulation of [^{3}H]colchicine.

Table 7.1 The effect of calphostin C and staurosporine on the accumulation of [^{3}H]daunorubicin and [^{3}H]colchicine in CEM and CEM/VLB cells

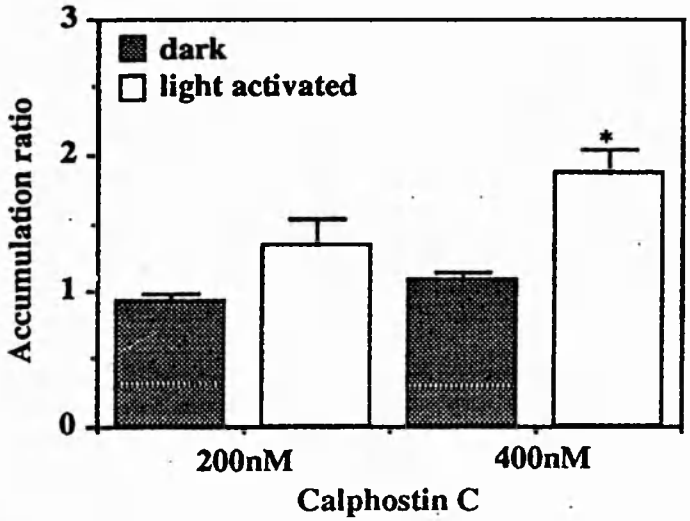
PKC INHIBITOR	DOSE	Accumulation ratio* [^{3}H]DAUNORUBICIN	Accumulation ratio* [^{3}H]COLCHICINE
Calphostin C(nM) (dark)	50	1.02 1.40	N/D
	100	1.19 1.90	N/D
	200	1.48** (0.08) n=6	1.16 1.45
	400	2.04* (0.23) n=5	1.44 (0.19) n=4
Calphostin C(nM) (light activated)	200	4.43** (0.25) n=3	3.20* (0.03) n=3
	400	8.82* (1.87) n=3	5.29* (1.30) n=3
Staurosporine(μM)	0.5	1.25 (0.22) n=3	1.49 1.73
	1.0	1.08 (0.07) n=3	1.14 (0.05) n=3
	2.0	1.48 (0.36) n=3	1.02 1.82
H7 (μM)	10.0	1.22 1.03	0.91 1.32
	20.0	1.52 1.16	0.92 1.72

*p<0.05 (significantly different from 1.0). **p<0.01 (highly significantly different from 1.0) Student's t test. Values are means of n independent experiments. Where n<3, individual values are shown. Parentheses show standard error. N/D- not determined

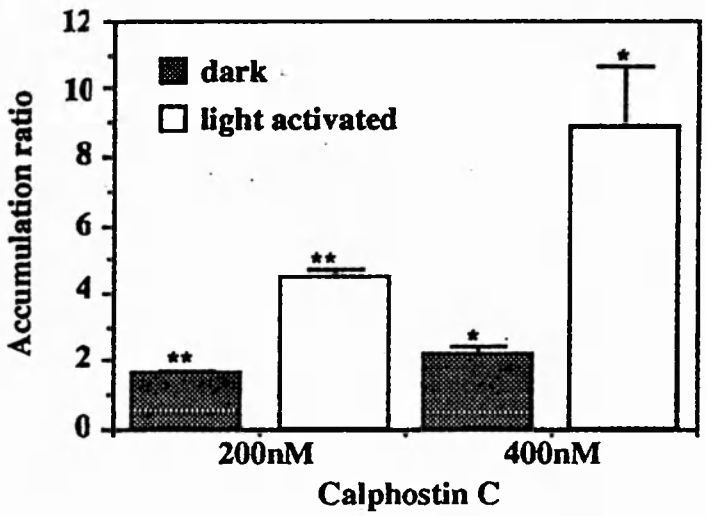
$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

Figure 7.1

(a) The effect of calphostin C (light and dark activated) on the accumulation of [^{3}H]daunorubicin in CEM cells



(b) The effect of calphostin C (light and dark activated) on the accumulation of [^{3}H]daunorubicin in CEM/VLB cells

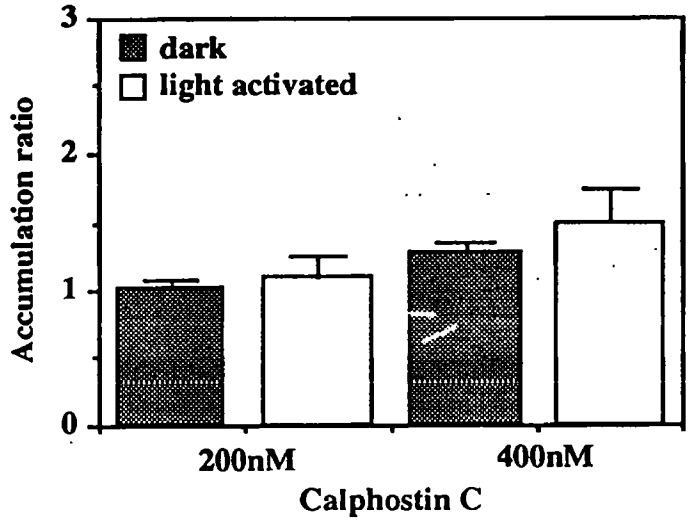


*p<0.05 (significantly different from 1.0). **p<0.01 (highly significantly different from 1.0), Student's t test. Values are means (standard error) of 3 independent experiments.

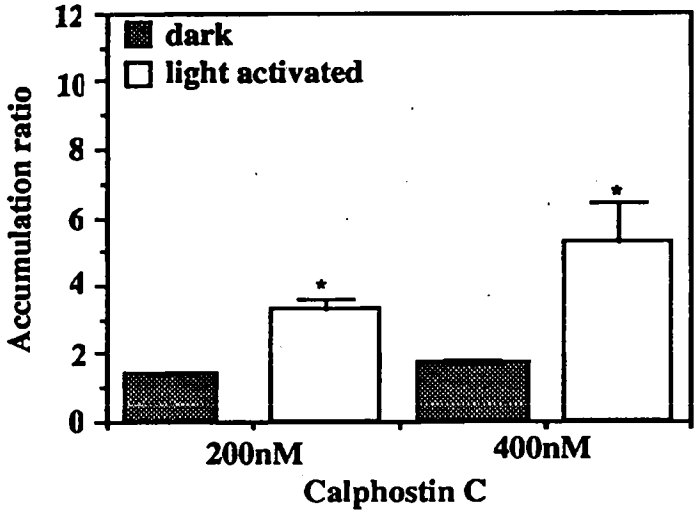
$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

Figure 7.2

(a) The effect of calphostin C (light and dark activated) on the accumulation of [^{3}H]colchicine in CEM cells



(b) The effect of calphostin C (light and dark activated) on the accumulation of [^{3}H]colchicine in CEM/VLB cells



*p<0.05 (significantly different from 1.0).

**p<0.01 (highly significantly different from 1.0. Student's t test)

Values are means (standard error) of 3 independent experiments.

$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

Figure 7.3 demonstrates that higher doses of calphostin C were able to increase accumulation in the resistant cell line, CEM/VLB without light activation. This effect may however be due to the toxicity of calphostin C, for example permeabilisation of the plasma membrane, at the doses used here and not to a direct effect on P-glycoprotein or other mechanisms causing the drug accumulation deficit seen in this cell line.

Figure 7.3

The effect of calphostin C (high dose, non light activated) on the accumulation of [^{3}H]daunorubicin in CEM/VLB cells

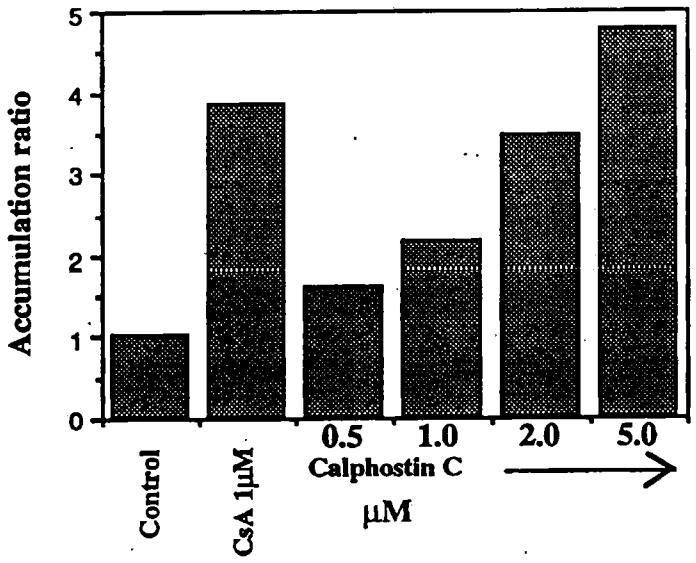


Figure represents a typical data set.

Similar results were obtained in 1 other independent experiment

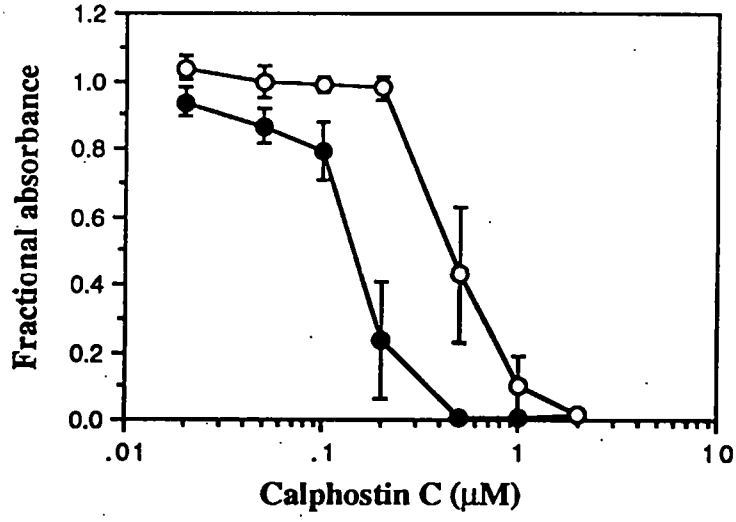
$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

7.3.2 Toxicity of calphostin C in CEM and CEM/VLB cells

Parent, CEM, and drug resistant, CEM/VLB, cells were exposed to calphostin C for a period of 2 hours in order to examine the acute toxic effects of the drug in these cell lines. Figure 7.4 indicates that the resistant cell line, CEM/VLB is slightly cross resistant to calphostin C. The mean IC_{50} (dose of cytotoxic drug required to reduce fractional absorbance to 50% of control values in the MTT assay) of calphostin C in CEM and CEM/VLB cells is 0.17μM and 0.49μM respectively (Table 7.2).

Figure 7.4

Toxicity of calphostin C (2h acute exposure, non-light activated) in CEM and CEM/VLB cells



Values are means (±standard error) of at least 3 independent experiments

(●) parent,CEM cells; (○) resistant,CEM/VLB cells

To determine the effect of light activation on the toxicity of calphostin C we again exposed parent, CEM and drug resistant, CEM/VLB cells to calphostin C for a period of 2 hours in the presence of either light activated or non-light activated calphostin C. Figure 7.5 shows that light activation clearly increases the toxicity of calphostin C to

both CEM and CEM/VLB cells. IC50 values obtained in 2 independent experiments are given in table 7.2.

Figure 7.5

Toxicity of calphostin C (2h acute exposure) in CEM/VLB cells (light versus dark)

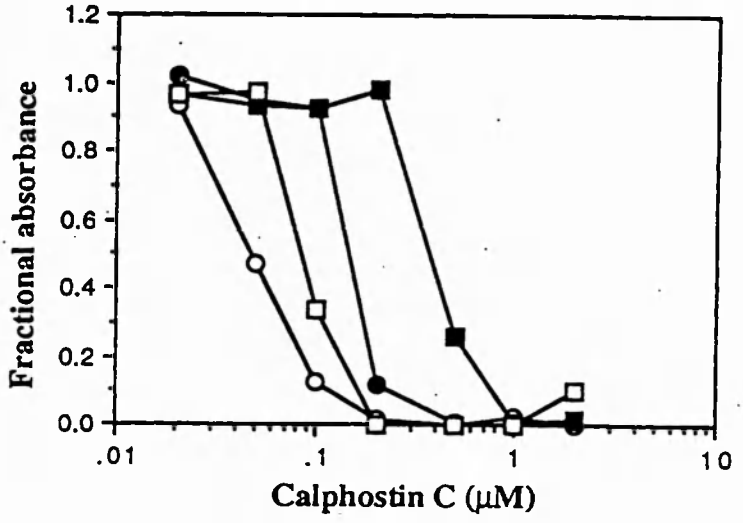


Figure represents a typical data set. Similar results were obtained in 2 independent experiments.

IC_{50} values for the 2 independent experiments are given in table 7.2.

(●)parent,CEM cells(dark); (○)parent,CEM cells (light)

(■)resistant,CEM/VLB cells(dark); (□)resistant,CEM/VLB cells (light)

Table 7.2

IC_{50}s for calphostin C (light and non-light activated) in CEM and CEM/VLB cells

Cell line	IC_{50} (μM) ±standard error
CEM (dark)	0.17±0.05
CEM/VLB (dark)	0.49±0.11*
CEM (light)	0.03 0.04
CEM/VLB (light)	0.08 0.13

*p=0.032 (IC_{50} value in resistant, CEM/VLB cell line is significantly different from the IC_{50} value in sensitive, CEM cell line, Student's t test). Values are means of n independent experiments. Where n<3, individual values are shown. IC_{50}=concentration of drug required to reduce fractional absorbance to 50% of control values in the MTT assay

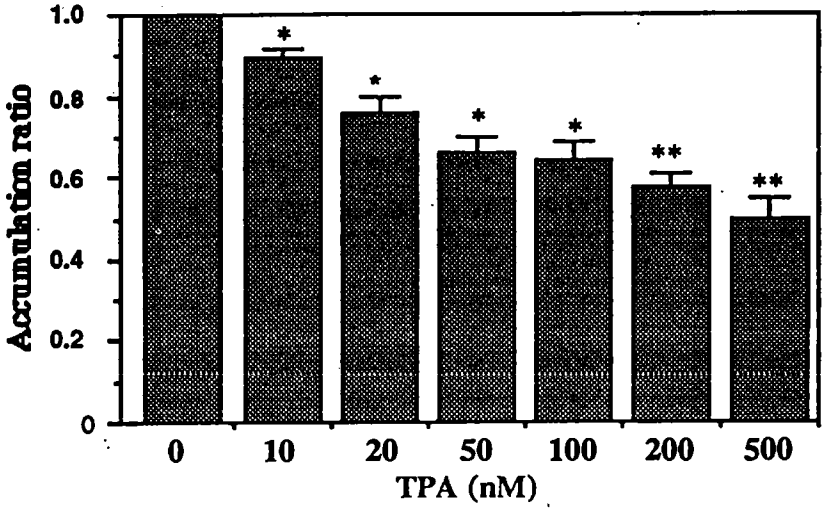
7.3.3 Effect of the protein kinase C activator, TPA on the accumulation of [^{3}H]daunorubicin and [^{3}H]colchicine in CEM and CEM/VLB cells

Previous studies have reported that enhancement of PKC activity by the phorbol ester TPA can increase drug transport in multidrug resistant cells (Fine *et al.*, 1988; Chambers *et al.*, 1990; Bates *et al.*, 1993). Since P-glycoprotein phosphorylation is enhanced by TPA treatment (Bates *et al.*, 1993), these results suggest that increasing PKC activity and therefore increasing P-glycoprotein phosphorylation may increase the drug transport capability of P-glycoprotein and therefore make the cell more resistant to cytotoxic drugs. In order to establish whether increasing PKC activity causes an increase in drug resistance we decided to look at the effect of TPA in the drug resistant cell line CEM/VLB. Cells were exposed to TPA for 30 minutes before the addition of the radiolabelled drug. The cells were then exposed to the radiolabelled drug for 1h in the continued presence of TPA. We observed that TPA caused a decrease in the accumulation of both [^{3}H]daunorubicin and [^{3}H]colchicine in

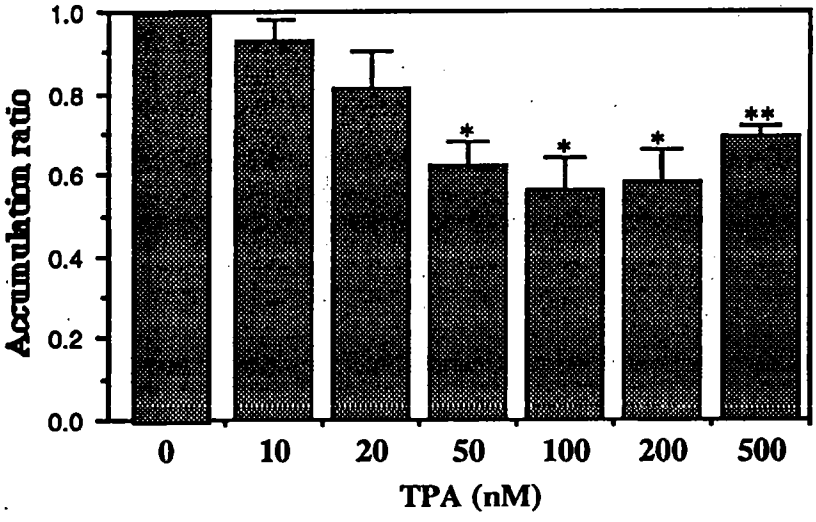
the drug resistant CEM/VLB cells. [^{3}H]Daunorubicin accumulation in CEM/VLB cells decreased with increasing doses of TPA (Figure 7.6a). The highest dose of TPA (500nM) caused a 50% decrease in [^{3}H]daunorubicin accumulation compared to the control ([^{3}H]daunorubicin accumulation in the absence of TPA). [^{3}H]Colchicine accumulation in CEM/VLB cells decreased with increasing doses of TPA until a dose of 100nM (Figure 7.6b). This dose of TPA caused a 57% decrease in [^{3}H]colchicine accumulation compared to the control ([^{3}H]colchicine accumulation in the absence of TPA). Higher doses of 200 and 500nM TPA did not cause [^{3}H]colchicine accumulation to decrease any further.

Figure 7.6

(a) Effect of TPA on the accumulation of [^{3}H]daunorubicin in CEM/VLB cells



(b) Effect of TPA on the accumulation of [^{3}H]colchicine in CEM/VLB cells



*p<0.05 (significantly different from 1.0). **p<0.01 (highly significantly different from 1.0), Student's t test. Values are means (standard error) of 3 independent experiments.

$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

Figure 7.7 shows that TPA was also able to reduce [^{3}H]colchicine accumulation induced by the treatment of the cells with cyclosporin A (a modifier of MDR). Cyclosporin A (1μM) was able to increase the accumulation of [^{3}H]colchicine in CEM/VLB cells to 2.5 times the level of the control ([^{3}H]colchicine accumulation in CEM/VLB cells in the absence of modifier). TPA (200nM) reduced this increase to 1.5 times the level of the control (Figure 7.6). Accumulation ratios for 2 independent experiments are shown in table 7.4.

Figure 7.7 Effect of TPA in combination with cyclosporin A on the accumulation of [^{3}H]colchicine in CEM and VLB cells

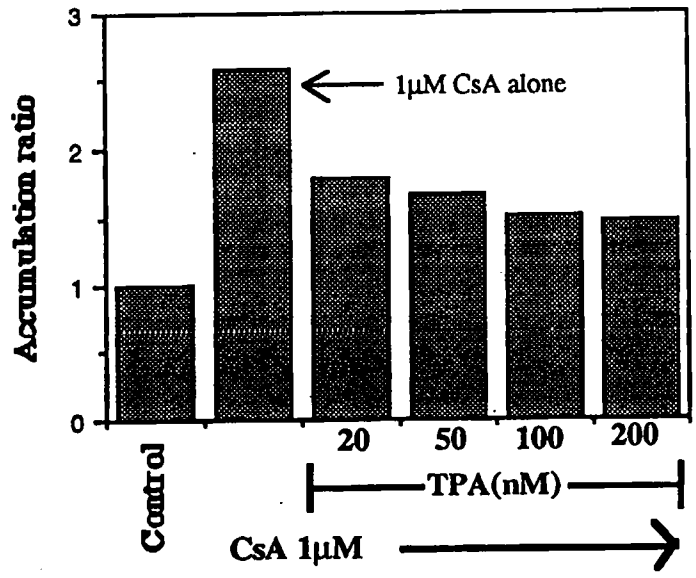


Figure represents mean of 2 independent experiments, independent values are shown in Table 7.3

$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

Table 7.3

Effect of TPA in combination with cyclosporin A on the accumulation of [^{3}H]colchicine in CEM and VLB cells

GROUP	Accumulation Ratio
Control	1.0 1.0 (1.0)
CsA (1μM) alone	2.79 2.37 (2.58)
CsA (1μM)+TPA (20nM)	2.01 1.57 (1.79)
CsA (1μM)+TPA (50nM)	1.77 1.57 (1.67)
CsA (1μM)+TPA (100nM)	1.62 1.40 (1.51)
CsA (1μM)+TPA (200nM)	1.50 1.46 (1.48)

Values shown are accumulation ratios from 2 independent experiments,

parentheses show means of 2 values.

Each individual value is the mean of triplicate determinations within each independent experiment.

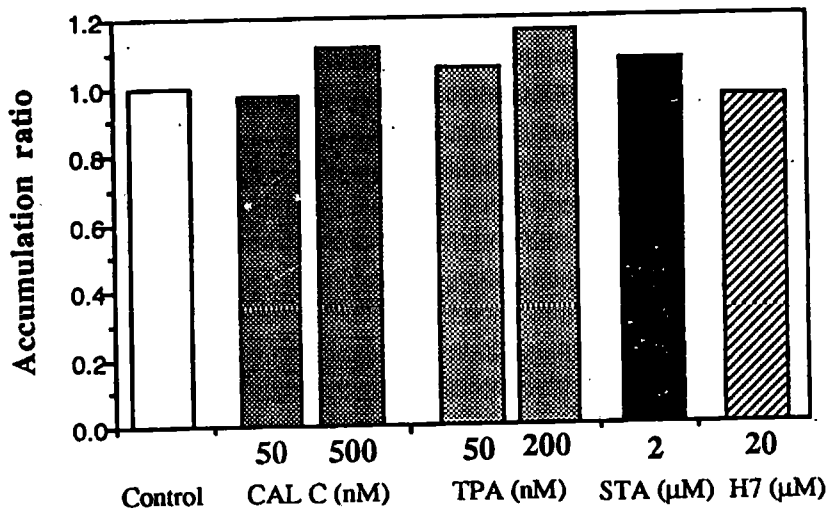
$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

7.3.4 Effect of calphostin C, TPA and staurosporine on the accumulation of [^{3}H]daunorubicin in CEM, drug sensitive cells

Figure 7.7. demonstrates that the PKC inhibitors, staurosporine, H7 and calphostin C have no effect on the accumulation of [^{3}H]daunorubicin in the drug sensitive CEM cell line. The PKC activator, TPA also has no effect on drug accumulation in this cell line. Results were similar in experiments to examine the effects of these agents on the accumulation of [^{3}H]colchicine in the CEM cell line (results not shown).

Figure 7.8

Effect of calphostin C, TPA and staurosporine on the accumulation of [^{3}H]daunorubicin in CEM, drug sensitive cells



Abbreviations: calphostin C (CAL C), staurosporine (STA)

Figure represents mean of 2 independent experiments. Individual values are shown in Table 7.4

$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

Table 7.4

Effect of calphostin C, TPA and staurosporine on the accumulation of [^{3}H]daunorubicin in CEM, drug sensitive cells

GROUP	Accumulation Ratio
Calphostin C (dark, 50nM)	0.98 0.98 (0.98)
Calphotin C (dark, 500nM)	1.17 1.05 (1.11)
TPA (50nM)	1.07 1.03 (1.05)
TPA (200nM)	1.21 1.11 (1.16)
Staurosporine (2μM)	1.09 1.03 (1.06)
H7 (20μM)	0.99 0.97 (0.98)

Values shown are accumulation ratios from 2 independent experiments,

parentheses show means of 2 values.

Each individual value is the mean of triplicate determinations within each independent experiment.

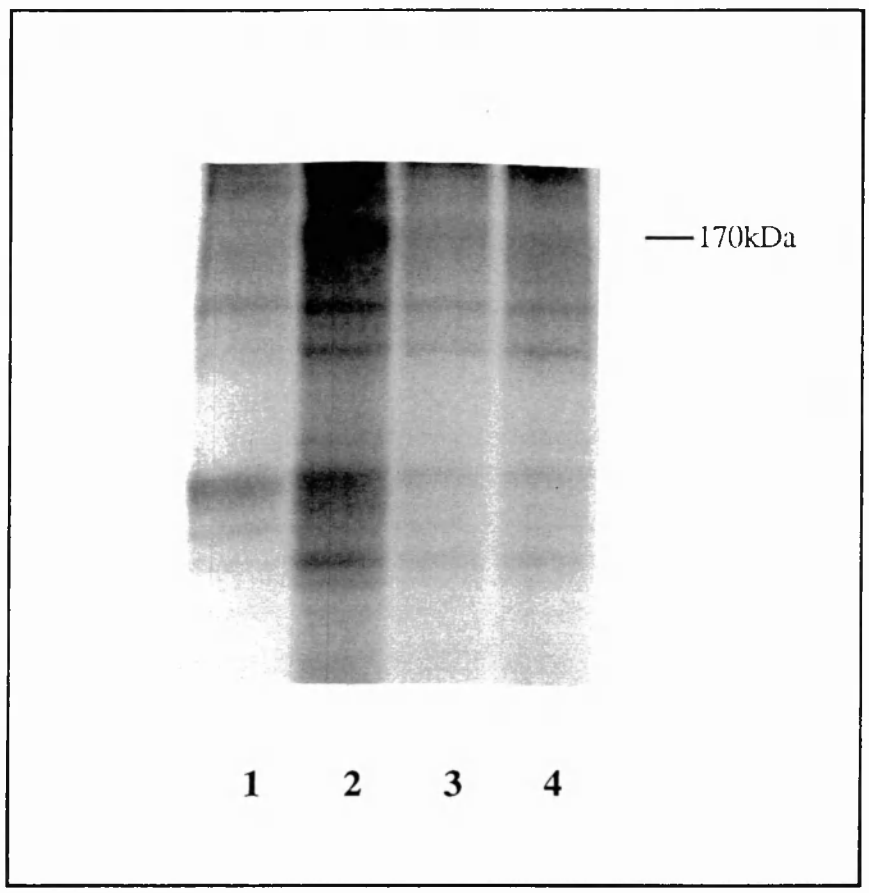
$$\text{Accumulation ratio} = \frac{[^3\text{H}]\text{DNR accumulation + modifier}}{[^3\text{H}]\text{DNR accumulation - modifier}}$$

7.3.5 Effect of the protein kinase C inhibitors, H7 and staurosporine on the phosphorylation of P-glycoprotein in cell membranes prepared from H69/P and H69/LX4 cells

In the search for the mechanism(s) underlying the effects of the PKC inhibitors on the transport of drugs in MDR cells, we decided to look at their effect on the phosphorylation of P-glycoprotein *in vitro* . These studies were undertaken in membranes prepared from the small cell lung cancer cell line H69/P and its MDR variant H69/LX4. These were the preliminary studies in this section of my research. After looking firstly at the effect of staurosporine and H7 on the phosphorylation of P-glycoprotein in these membranes we decided that it would be more appropriate to look at the effect of PKC inhibitors in an intact cell system as we were, after all, performing all drug accumulation studies in whole cells and not membrane vesicles. We decided, therefore, to carry on our studies using a intact cell phosphorylation assay. We elected to use the CEM and CEM/VLB cell lines: (a) because this is the cell line that we used in the accumulation studies and we wanted to be able to make direct comparisons and (b) because these cell lines grow as single cell suspensions in comparison to the H69/P and H69/LX4 cells lines which grow as floating aggregates. Figure 7.9 shows the effects of the protein kinase C inhibitors, H7 and staurosporine on the phosphorylation of P-glycoprotein in cell membranes prepared from MDR, H69/LX4 cells. In lane 2, there is a clear band which represents the phosphorylation of P-glycoprotein in membranes prepared from the P-glycoprotein overexpressing cell line, H69/LX4. In contrast there is no band in lane 1, the parental cell line which does not express P-glycoprotein. Lanes 3 and 4 show the inhibition of phosphorylation of P-glycoprotein by H7 10μM and staurosporine 2μM respectively.

Figure 7.9

Effects of the protein kinase C inhibitors, H7 and staurosporine on the phosphorylation of P-glycoprotein in cell membranes prepared from MDR, H69/LX4 cells.



Lane 1, H69/P; Lane 2, H69/LX4; Lane 3 H69/LX4 + H7 10μM;

Lane 4 H69/LX4 + staurosporine 2μM

7.3.6 Effect of protein kinase C inhibitors and the protein kinase C activator, TPA on the on the phosphorylation of P-glycoprotein in intact MDR, CEM/VLB cells

The effects of the non specific, PKC inhibitors staurosporine and H7 on the phosphorylation of P-glycoprotein in intact CEM/VLB cells are shown in figure 7.10 Lanes 1 and 2 represent the parent, CEM, and resistant, CEM/VLB cell lines respectively. In Lane 2 the presence of a band at a molecular weight of 170kDa can be observed (molecular weight markers were run along side the proteins). This band represents the phosphorylation of P-glycoprotein. This band of P-glycoprotein is absent in the parental cell line, CEM. Lanes 3 and 4 represent the effects of staurosporine (2μM) and H7 (10μM) respectively on the phosphorylation of P-glycoprotein in CEM/VLB cells. Staurosporine and H7 do not appear to inhibit phosphorylation in this MDR cell line.

Figure 7.10

Effect of staurosporine and H7 on the phosphorylation of P-glycoprotein in intact MDR, CEM/VLB cells

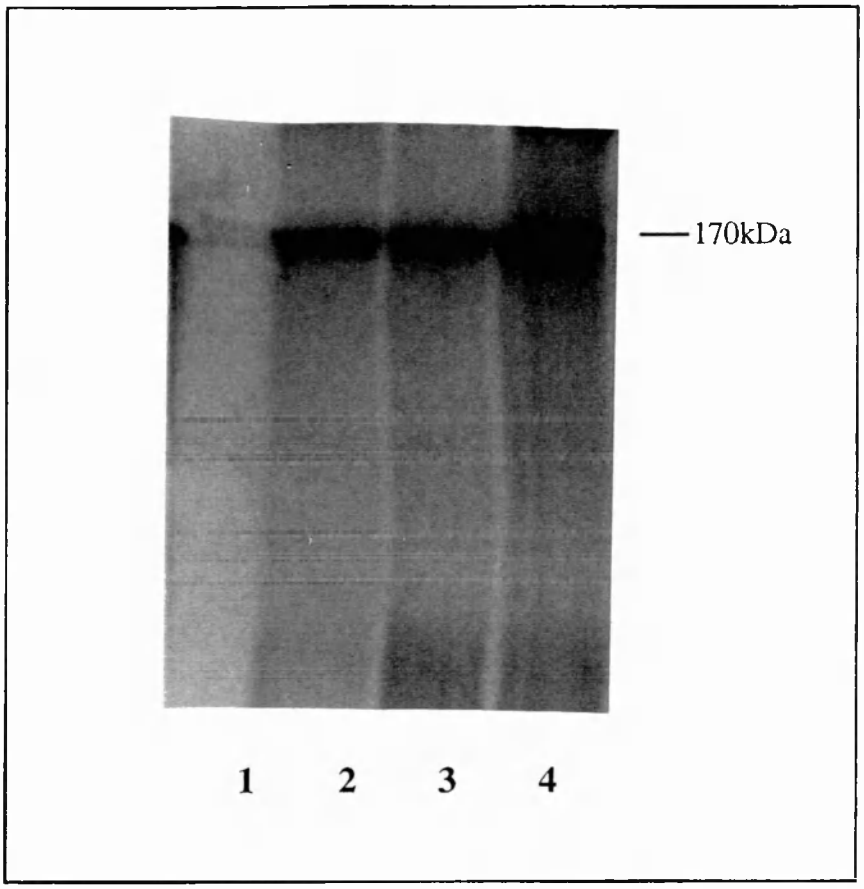


Figure 7.14 demonstrates the difference in effect of light- and non-light-activated calphostin C on the phosphorylation of P-glycoprotein in intact CEM and CEM/VLB cells. Again no band of phosphorylation is observed in Lane 1, the parental CEM cells line. In contrast in the P-glycoprotein overexpressing cell line, CEM/VLB (lane 2) a band of phosphorylation is observed at a molecular weight of 170kDa (the molecular weight of P-glycoprotein. Lanes 3, 4 and 5 represent the effect of calphostin C (200nM:light), calphostin C (200nM:dark) and 200nM TPA respectively on the phosphorylation of P-glycoprotein in CEM/VLB cells. This gel photograph clearly shows that light activated calphostin C was able to inhibit the phosphorylation of P-glycoprotein. In contrast, the same concentration of calphostin C without light activation had no effect. In lane 5 TPA, however, the density of the phosphorylated band at 170kDa appears to be increased. Thus TPA increased the phosphorylation of P-glycoprotein in CEM/VLB cells.

Figure 7.11

Effect of calphostin C (light and dark activated) and TPA on the phosphorylation of P-glycoprotein in intact MDR, CEM/VLB cells

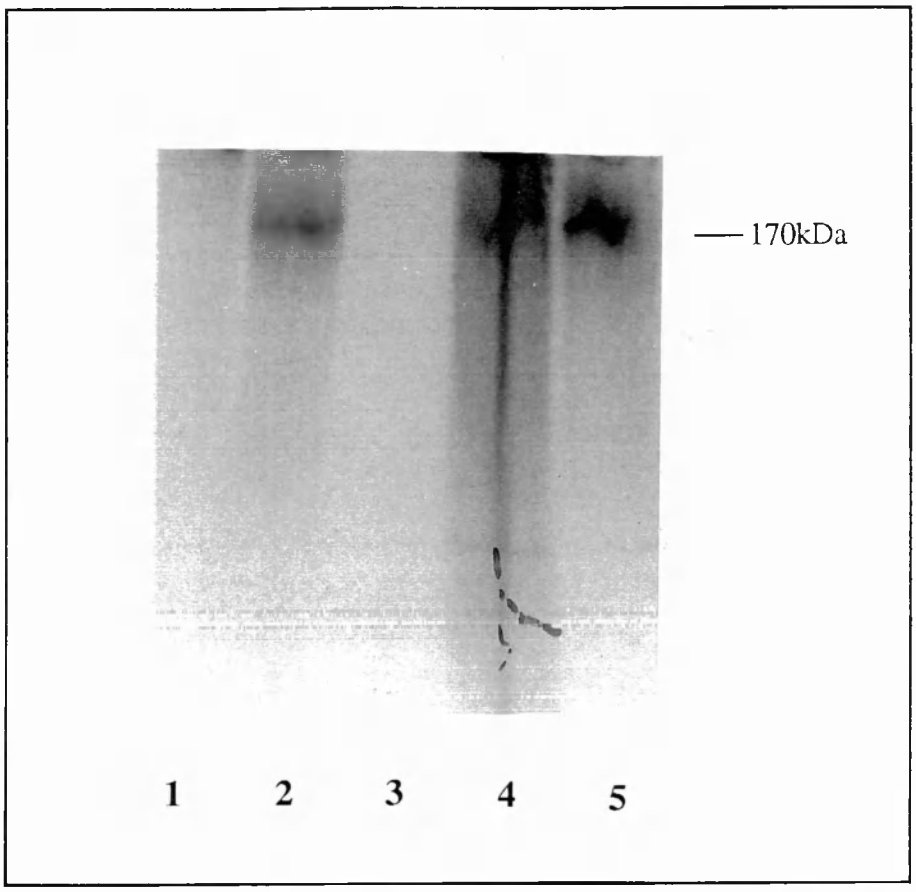
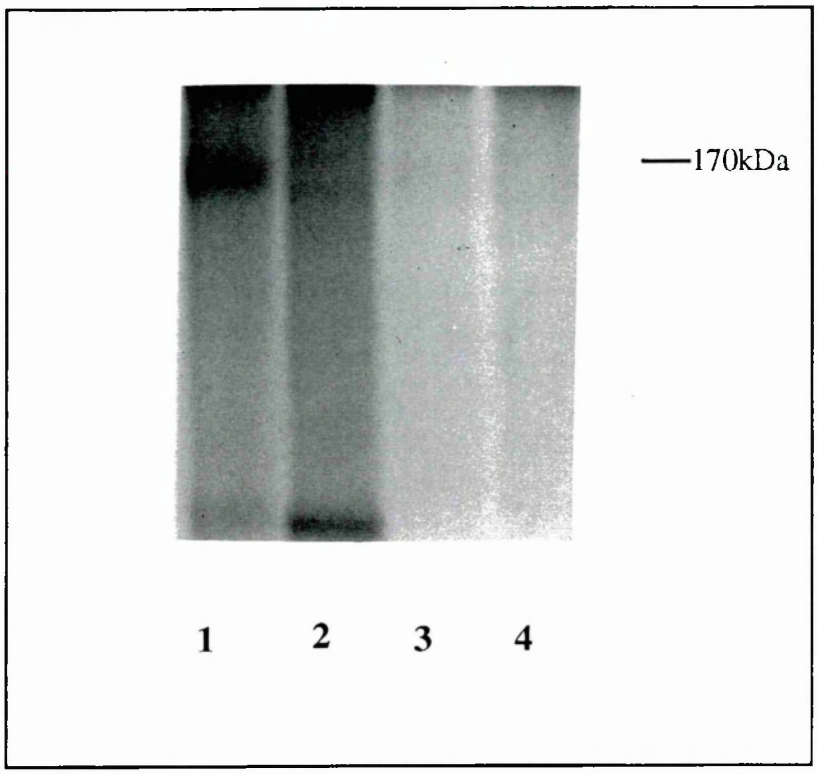


Figure 7.12 again demonstrates the effect of light activated calphostin C on the phosphorylation of P-glycoprotein. In lanes 3 and 4 (CEM/VLB, resistant cells) light activated calphostin C at 200 and 400nM respectively caused a complete loss of the band of phosphorylation observed at a molecular weight of 170kDa in lane 1 (CEM/VLB, resistant cells). In comparison there is no band in lane 2 which represents the drug sensitive parental cell line, CEM.

Figure 7.12

Effect of calphostin C (light activated) on the phosphorylation of P-glycoprotein in intact MDR, CEM/VLB cells

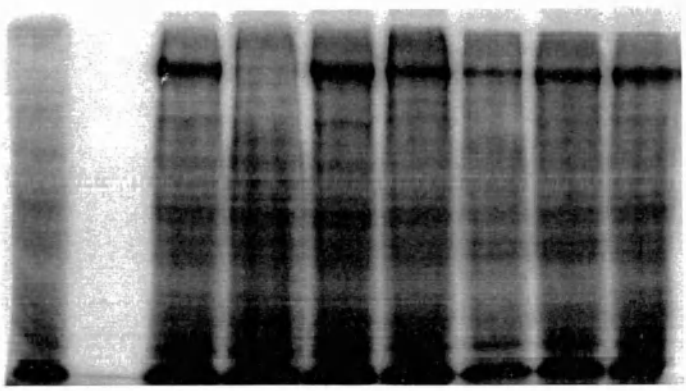


7.3.7. Effect of protein kinase C inhibitors on the ability of photo-labelled [^{3}H]azidopine to bind to P-glycoprotein in membranes prepared from CEM/VLB cells

The gel photograph shown in Figure 7.13 shows the effect of cyclosporin A, calphostin C (dark), staurosporine and H7 on the ability of [^{3}H]azidopine to photo label P-glycoprotein in membranes prepared from CEM/VLB cells. Lane 1 shows no azidopine binding and represents the parental CEM cell line. Lane 2 is a control and represents CEM/VLB membranes after exposure to [^{3}H]azidopine but without exposure to UV light. As [^{3}H]azidopine requires photoactivation no binding occurs. Lane 3 shows the presence of a band at a molecular weight of 170kDa representing [^{3}H]azidopine binding to P-glycoprotein. The [^{3}H]azidopine binding to P-glycoprotein was inhibited by 5μM cyclosporin A (lane 4). Lanes 5, 6, 7, 8 and 9 show the effect of calphostin C (dark) 200nM, calphostin C (dark) 400nM, staurosporine 2μM, H7 10μM and H7 20μM on the ability of [^{3}H]azidopine to photolabel P-glycoprotein in CEM/VLB cells. Only staurosporine 2μM in lane 7 shows any inhibition of [^{3}H]azidopine photolabelling.

Figure 7.13

Effect of H7, staurosporine and calphostin C (dark) on the ability of photo-labelled [^{3}H]azidopine to bind to P-glycoprotein in membranes prepared from CEM/VLB cells



Lane number 1 2 3 4 5 6 7 8 9

Legend:

Lane 1; CEM (parent),

Lane 2; CEM/VLB (resistant) no UV light,

Lane 3; CEM/VLB no modifier,

Lane 4; CEM/VLB + cyclosporin A 5μM,

Lane 5 & 6; CEM/VLB + Calphostin C 200 & 400nM respectively,

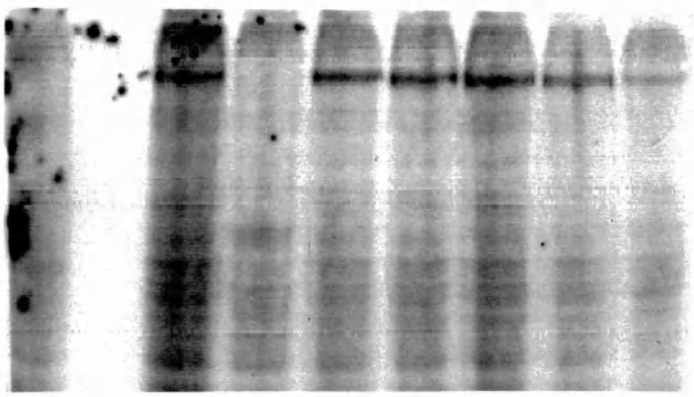
Lane 7; CEM/VLB + staurosporine 2μM,

Lane 8 & 9; CEM/VLB + H7 10μM & H7 20μM

In figure 7.14 lanes 1to 4 are the same as for figure 7.13. Lanes 5, 6, 7, 8 and 9 represent the effect of calphostin C (dark) 200nM, 500nM, 1μM, 2μM and 5μM respectively. At the higher doses of 2 and 5μM the [^{3}H]azidopine photolabelling of P-glycoprotein was partially inhibited.

Figure 7.14

Effect of increasing doses of calphostin C (dark) on the ability of photo-labelled [^{3}H]azidopine to bind to P-glycoprotein in membranes prepared from CEM/VLB cells



Lane number 1 2 3 4 5 6 7 8 9

Legend:

Lane 1; CEM (parent),

Lane 2; CEM/VLB (resistant) no UV light,

Lane 3; CEM/VLB no modifier,

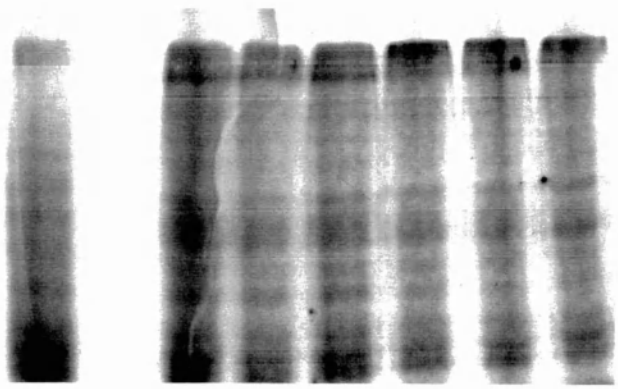
Lane 4; CEM/VLB + cyclosporin A 5μM,

Lane 5, 6, 7, 8 & 9; CEM/VLB + Calphostin C (dark) 0.2, 0.5, 1.0, 2.0, 5.0 μM respectively

The difference in the effect of light and non-light activated calphostin C is demonstrated by the gel photograph shown in figure 7.15. Lanes 1to 3 are the same as for figure 7.13. Lanes 4, 5, 6, 7, and 8 represent the effect of calphostin C (dark) 200nM, 400nM, calphostin C (light-activated) 200nM, 400nM respectively. As shown previously (Figure 7.12) calphostin C (dark) at concentrations of 200 and 400nM had no effect on the ability of [^{3}H]azidopine to photolabel P-glycoprotein. It appears that 200 and 400nM calphostin C(light-activated) did, however, cause inhibition of the [^{3}H]azidopine to photolabelling.

Figure 7.15

Effect of calphostin C (light and dark activated) on the ability photo-labelled [^{3}H]azidopine to bind to P-glycoprotein in membranes prepared from CEM/VLB cells



Lane number 1 2 3 4 5 6 7 8

Legend:

Lane 1; CEM (parent),

Lane 2; CEM/VLB (resistant) no UV light,

Lane 3; CEM/VLB no modifier,

Lane 4; CEM/VLB + cyclosporin A 5μM,

Lanes 5 & 6; CEM/VLB + calphostin C (dark) 200nM & 400nM respectively,

Lanes 7 & 8; CEM/VLB + Calphostin C (light-activated) 200nM & 400nM respectively.

7.4 Discussion

The studies presented here extend earlier observations of alterations in P-glycoprotein function after treatment of multidrug resistant cells with agents that interfere with P-glycoprotein phosphorylation. Inhibition of PKC with calphostin C decreased the phosphorylation of P-glycoprotein and increased the accumulation of daunorubicin and colchicine in the P-glycoprotein overexpressing MDR cells, CEM/VLB.

Several groups have shown that P-glycoprotein is phosphorylated (Carlsen *et al.*, 1977; Center, 1983; Hamada *et al.*, 1987). Tsuruo demonstrated that TPA, verapamil and trifluoperazine could modulate P-glycoprotein phosphorylation (Hamada *et al.*, 1987). Subsequently, Fine and Chambers demonstrated increased drug resistance after treatment with the phorbol ester, TPA (Fine *et al.*, 1988; Chambers *et al.*, 1990). Chambers observed that this increase in drug resistance was mediated by decreased vinblastine accumulation and was associated with an increase in P-glycoprotein phosphorylation. Bates *et al.* (1992) confirmed the relationship between increased P-glycoprotein phosphorylation and enhanced P-glycoprotein mediated vinblastine efflux after TPA treatment. Other studies associated inhibition of P-glycoprotein phosphorylation by staurosporine with decreased drug efflux (Ma *et al.*, 1991; Sato *et al.*, 1990; Sampson *et al.*, 1993). Our results indicate that staurosporine has no significant effect on drug transport in the sensitive, CEM or the drug resistant, CEM/VLB cell lines. Interestingly, although staurosporine did not appear to inhibit the phosphorylation of P-glycoprotein in the intact drug resistant, CEM/VLB cells, it did inhibit the phosphorylation of P-glycoprotein in membranes prepared from another MDR P-glycoprotein overexpressing cell line, H69/LX4. This may indicate that either the actions of staurosporine are cell specific or that staurosporine is able to inhibit particular PKC isoenzymes with a greater potency than others. In intact cells, endogenous protein kinases that are not associated with the membrane are still available to phosphorylate P-glycoprotein. If staurosporine is not specific for these enzymes but is able to inhibit the membrane associated protein kinases in the

H69/LX4 cell membranes this may explain the apparent difference in activity of staurosporine in these two systems. Bates *et al.* (1993) observed that calphostin C affects PKC z and not a and that high levels of PKC z were located in the membrane fraction. Similar specific isoenzyme studies need to be carried out with staurosporine in order to explain the differences observed in this study. Staurosporine is an indole carbazole produced by a *Streptomyces* species which inhibits PKC and a variety of other kinases with high potency. The IC_{50} (concentration required for 50% inhibition) for inhibition of PKC is 6nM (O'Brian *et al.*, 1990). If staurosporine is able to modulate the function of P-glycoprotein by inhibiting its phosphorylation, it should be capable of doing so at doses in the nanomolar range. Looking retrospectively at previous studies, Ma *et al.* (1991) found that a dose of 1μM staurosporine partially restored the daunomycin accumulation in the vincristine resistant, P-glycoprotein overexpressing cell line, HL60/Vinc. We also used this concentration, as we found it to be non toxic in the CEM and CEM/VLB cell lines. Sato *et al.*, however, used a concentration of 20μM. There is no doubt that these relatively high concentrations of staurosporine should inhibit PKC and thus the phosphorylation of P-glycoprotein. Staurosporine itself exhibits a wide range of toxic effects against various cultured cells. At high doses such as those used in the study by Sato *et al.*, the possibility of toxic effects can not be ruled out. It is possible that staurosporine may reduce the phosphorylation of P-glycoprotein by slowing down the incorporation of orthophosphate to ATP rather than (or in addition to) acting via the inhibition of PKC. Sato *et al.* also noted that staurosporine inhibited the photoaffinity labelling of P-glycoprotein with [^{3}H]azidopine indicating that staurosporine may competitively inhibit drug binding to P-glycoprotein by itself binding to the protein. Our results confirm this. As shown in Figure 7.11 and discussed in Section 7.3.7, 1μM staurosporine partially inhibits the binding of [^{3}H]azidopine to P-glycoprotein in membranes prepared from CEM/VLB cells. Further studies using low, non toxic doses of staurosporine in MDR cell lines known to be affected by the drug will determine whether the effect of staurosporine on drug

transport is via the inhibition of PKC or via a direct interaction with P-glycoprotein. Like staurosporine, the non specific PKC inhibitor H7 also inhibited the phosphorylation of P-glycoprotein in membranes prepared from drug resistant CEM/VLB cells but not in intact drug resistant CEM/VLB cells. H7 also had no effect on drug accumulation in either the parental CEM or drug resistant CEM/VLB cells.

In addition to the controversy surrounding the action of staurosporine in MDR cell lines, calphostin C has also been the subject of intense research for many groups. Gupta *et al.* (1994) proposed that calphostin C, at a concentration that increased drug accumulation and partially reversed drug resistance (250nM) did not inhibit PKC activity either in the cytosol or membrane fractions. These results contrast with those of Bates *et al.* who found that calphostin C at a concentration of 200nM inhibited PKC z in the membrane fraction of MDR, SW620Ad300 cells. Our results confirm that light activated calphostin C inhibits the phosphorylation of P-glycoprotein and in turn modulates drug transport in the drug resistant cell line, CEM/VLB. The inhibition of PKC in intact cells by calphostin C has been shown to require light (Bruns *et al.*, 1991). Our results demonstrate that P-glycoprotein phosphorylation is only inhibited by light activated calphostin C. In experiments carried out under dark conditions, calphostin C had no effect on the phosphorylation of P-glycoprotein. It appears that calphostin C may inhibit P-glycoprotein phosphorylation by light-dependent inhibition of PKC. Several groups have examined the effect of calphostin C on the transport of cytotoxic drugs in MDR cells (Bates *et al.*, 1993; Gupta *et al.*, 1994). These groups have not, however, published data on the difference between the effects of light and dark activated calphostin C on drug transport in MDR cells. Our data suggests that non-light activated calphostin C significantly increases drug accumulation in the MDR cell line, CEM/VLB without any effect on the phosphorylation status of P-glycoprotein. This may suggest that calphostin C is able to reverse MDR by a mechanism other than via inhibition of P-glycoprotein

phosphorylation due to its ability to irreversibly inhibit PKC. Interestingly, light activated calphostin C has a much greater effect on drug accumulation. The increases in daunorubicin and colchicine accumulation observed in the presence of light activated calphostin C were 3- to 4-fold greater than those observed in the presence of non-light activated calphostin C. There are several possible explanations that may account for the difference in the effects of light activated and non-light activated calphostin C. Firstly, the increased ability of light activated calphostin C to modulate drug transport in MDR cells may be due to the inhibition of PKC by light activated calphostin C which in turn causes P-glycoprotein to become dephosphorylated with the result of a possible alteration in its function. Secondly, our data indicates that light activation enhances the toxicity of calphostin C in the drug resistant CEM/VLB cell line. Like staurosporine, calphostin C may reduce the phosphorylation of P-glycoprotein by slowing down the incorporation of orthophosphate to ATP rather than (or in addition to) acting via the inhibition of PKC. The increased toxicity may cause an increase in drug accumulation in CEM/VLB cells by permeabilising the membranes. In the presence of light calphostin C is known to react with molecular oxygen, generating singlet oxygen and forming endoperoxides (Bruns *et al.*, 1991). Membrane destruction resulting from lipid peroxidation by singlet oxygen is thought to be the major cause of toxicity of calphostin C in mammalian cells. This may explain the increased drug accumulation observed in cells exposed to light activated calphostin C. This, however, does not explain why seemingly toxic doses of calphostin C do not increase drug accumulation in the drug sensitive, CEM cell line to the same degree as in the drug resistant, CEM/VLB cell line.

The results of our photoaffinity labelling experiments demonstrate that high doses of calphostin C inhibit the [^{3}H]azidopine photolabelling of P-glycoprotein. This is in agreement with the results of Bates *et al.* (1993) who also suggest that this inhibition at high dose may be the result of a change in the conformation of P-glycoprotein due to the effect of calphostin C on the phosphorylation status of the protein. We

observed no inhibition of [^{3}H]azidopine labelling of P-glycoprotein in the presence of non-light activated calphostin C at doses used in our phosphorylation studies. However after light activation we did observe an inhibition of [^{3}H]azidopine labelling of P-glycoprotein. As the light activated form of calphostin C inhibits PKC it is possible that it may prevent [^{3}H]azidopine from labelling P-glycoprotein by altering its conformation by dephosphorylating the protein. This is in agreement with the theory of Bates *et al.* as discussed above. The results we obtained here do not prove that calphostin C causes an alteration in the drug binding capability of P-glycoprotein by dephosphorylating the protein but merely provide evidence to support the theory. The results presented here using the PKC activator TPA appear to agree with the findings of previous studies (Fine *et al.*, 1988; Chambers *et al.*, 1990; Bates *et al.*, 1993). The results of our phosphorylation experiments appear to indicate that TPA does increase the phosphorylation of P-glycoprotein, although further experiments are required to demonstrate a clear stepwise increase in P-glycoprotein phosphorylation with increasing doses of TPA. The accumulation studies presented here show that TPA reduces the intracellular accumulation of both [^{3}Hdaunorubicin and [^{3}H]colcicine in the MDR cell line, CEM/VLB after an incubation time of one hour. TPA activates PKC and therefore causes P-glycoprotein to become hyperphosphorylated. The decrease in drug accumulation observed in the presence of TPA may indicate that either hyperphosphorylated P-glycoprotein is a more efficient drug efflux pump or that TPA interferes in someway with the passive diffusion of drugs across the cell membrane.

If the state of phosphorylation does modulate the affinity of P-glycoprotein for various substrates, and in turn their transport, one could also predict differential effects on the ability of P-glycoprotein antagonists to block transport. Experiments demonstrating a differential effect of calphostin C on P-glycoprotein antagonism by verapamil and cyclosporin carried out by Susan Bates' group are consistent with this. The group predicts that some antagonists would be more effective against

phosphorylated P-glycoprotein and others against dephosphorylated P-glycoprotein. This theory is also consistent with previous observations of synergy with combinations of antagonists, including verapamil and cyclosporin A (Hu *et al.*, 1990) and quinine and verapamil (Lehnert *et al.*, 1991). Understanding the basis of this regulatory pathway may provide insight into approaches for developing new strategies for reversing drug resistance.

Some of the published data on the effect of PKC inhibitors on drug transport in MDR cells are contradictory and confusing. The PKC activity and other actions of PKC inhibitors with respect to P-glycoprotein (i.e. ability to bind directly to P-glycoprotein) must be considered when interpreting existing data.

In summary, this study demonstrates that the light activated form of the PKC inhibitor calphostin C has a greater effect on the transport of cytotoxic drugs in the MDR cell line CEM/VLB than the non-light activated form. This may indicate that P-glycoprotein-mediated drug efflux is modulated by phosphorylation. In the case of azidopine it appears that this modulation is a consequence of decreased binding, suggesting a mechanism which involves altered function of P-glycoprotein possibly due to dephosphorylation of the protein.

Chapter 8

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8.1 Introduction

Inevitably during the course of my 3 years of work at the Clinical Oncology and Radiotherapeutics Unit in Cambridge the goals of my work have changed. Initially the intention was for my studies to centre around the Xenova compounds, investigating their activity and mechanism(s) of action in cells which overexpress P-glycoprotein. However, as the screen for novel fungal product drug resistance modifiers at Xenova proved difficult to set up due to technical problems, I began initially to look at the activity of deoxyspergualin, a synthetic derivative of the antitumour antibiotic spergualin, in MDR cells. This enabled me to present work at meetings and to write my first scientific paper without the constraints of confidentiality that are associated with the Xenova compounds. The results of these studies are presented in Chapter 3.

While setting up the screen at Xenova, scientists identified derivatives of a natural product diketopiperazine which displayed promising activity as resistance modifiers. I selected 10 of these compounds as the basis of the structure-activity and mechanistic studies presented in Chapter 4.

In parallel with studies on P-glycoprotein-overexpressing cells, I routinely examined the activity of the Xenova compounds in the drug sensitive cell line L23/P and its MRP-overexpressing sub line L23/R. One of the initial 10 compounds showed activity as a resistance modifier in the L23/R cell line. Scientists at Xenova then produced 10 derivatives of this compound. We then examined the resistance modifying activity of these 10 new compounds in MRP-overexpressing MDR cells with the aim of finding a potent modifier of MRP-mediated MDR. One compound, XR9173, was clearly the most effective amongst the 10 compounds. The results of these studies are discussed in Chapter 5. In Chapter 6 we went on to examine the possible mechanisms of action of XR9173 by

examining its effect on calcein (an anionic fluorescent dye) accumulation and efflux, intracellular drug distribution, and GSH levels.

We observed that, at doses where some of the Xenova compounds were effective modifiers of P-glycoprotein-mediated MDR, they did not inhibit the photolabelling of P-glycoprotein with [^{3}H]azidopine. This may suggest that the mechanism of action of these compounds is one other than competitive binding to P-glycoprotein. Many groups have shown that P-glycoprotein is phosphorylated and that it is a substrate for PKC. We therefore decided to examine the effect of the XR compounds on the phosphorylation of P-glycoprotein. Meanwhile scientists at Xenova tested the ability of the compounds to inhiibit PKC and found that none of the compounds tested inhibited PKC. A US company called Novascreen also tested the compounds in a series of receptor binding assays. In a test for the ability of the compounds to inhibit the binding of the phorbol ester PDBu to PKC all the compounds were inactive. It is, therefore, unlikely that the Xenova compounds effect drug resistance via modifying the phosphorylation status of P-glycoprotein. We had, at this stage, already set up the phosphorylation assay. This assay proved difficult to set up due to a problem with identifying the most appropriate antibody to use for immunoproecipitation. In the initial phase of the assay we chose to test the assay by examining the effect of known PKC inhibitors on the phosphorylation of P-glycoprotein. We observed a diffence in the effect of light- and dark-exposed calphostin C (a specific inhibitor of PKC) and so, despite the fact that it no longer seemed appropriate to use the assay to examine the mechanism of action of the Xenova compounds, we were able to use it to investigate the role of phosphorylation in the function of P-glycoprotein. These studies are described in Chapter 7.

This final chapter summaries and highlights the main themes of the results obtained in each chapter.

8.2 The effect of the immunosuppressive, antibiotic, deoxyspergualin in drug sensitive and resistant cells

8.2.1 The toxicity of polyamines and deoxyspergualin in the presence and absence of copper amine oxidase in serum

The study presented in Chapter 3 supports the theory that aminoaldehydes and hydrogen peroxide produced by the oxidative deamination of polyamines by copper amine oxidases are responsible for the cytotoxicity of polyamines and deoxyspergualin *in vitro* in the presence of bovine serum. Our results using horse serum, however, which is low in copper amine oxidase content indicate that this may not be the only mechanism of toxicity. In agreement with other groups (Kuramochi *et al.* 1987; Shiro *et al.*, 1992) we found that deoxyspergualin was still toxic even in the absence of serum containing copper amine oxidase. Also in agreement with the work of Kuramochi *et al.* (1987) our survival curves indicate that in the absence of copper amine oxidase the mechanism of action of deoxyspergualin may be cytostatic. and in the presence of copper amine oxidase the mechanism of action may be cytotoxic. Nishikawa *et al.*, (1991) showed that deoxyspergualin arrested cells in G_0/G_1 phase and reduced the cycling cell population. The conversion of G_0 to G_1 phase and progression to S phase are two critical steps for cell proliferation. The cytostatic effect of deoxyspergualin in the absence of copper amine oxidase is, therefore, reflected by the drugs' effect on the cell cycle.

8.2.2 Deoxyspergualin is not an effective modifier of multidrug resistance

Cyclosporin A and FK506 are natural products possessing potent immunosuppressive properties and also the ability selectively to restore sensitivity to cells with acquired multidrug resistance. We were able to demonstrate that deoxyspergualin does not inhibit the ability of [^{3}H]azidopine to photolabel P-glycoprotein suggesting that it is not a substrate for the protein. This is supported by

the fact that deoxyspergualin does not reverse the accumulation deficit in EMT6/AR1.0 or H69/LX4 cells and that it is not capable of restoring the sensitivity of the drug resistant cell lines EMT6/AR1.0 or H69/LX4. These results indicate that deoxyspergualin does not modify classical multidrug resistance. The maintenance of activity in classical multidrug resistant cells and its potent *in vivo* anti-tumour activity, however, makes deoxyspergualin a promising agent for further investigations into its potential clinical use as an antitumour agent.

8.3 Xenova compounds as modifiers of P-glycoprotein-mediated multidrug resistance

8.3.1 Introduction

In Chapter 4 our aim was to estabish the activity of a group of noval fungal product diketopiperazines as modifiers of P-glycoprotein-mediated multidrug resistance and to build up stucture activity relationships for the compounds. For those compounds that showed activity as resistance modifiers we have attempted to elucidate their mechanism of action.

8.3.2 The most effective modifiers of P-glycoprotein mediated multidrug resistance contain a tetrahydroisoquinoline within their structure

All the molecules with the exception of XR9089 contain a diketopiperazine within their structures. Compounds containing this structure have previously been shown to be active resistance modifiers (Kamei *et al.*, 1990). Compounds XR9006, XR9051, XR9019 and XR9112 were the most active modifiers of P-glycoprotein-mediated MDR. These compounds contain a tetrahydroisoquinoline within their structures. This makes the molecules protonated at physiological pH and may explain why these compound are more efficient modifiers. Similarly other known modifiers of MDR, for example verapamil, contain the same functional group. Compounds that contain no positive charge at physiological pH , for example

XR1500, were less effective modifiers of P-glycoprotein-mediated multidrug resistance. The two most active compounds, XR9051 and XR9006 have different acivities depending on the cytotoxic drug with which they are used in combination. The only difference in their structures is that the side chain containing the tetrahydroisoquinoline group is in the meta position on the benzylidene ring in XR9051 and in the para position in XR9006. This difference in positioning of the tetrahydroisoquinoline group will serve to alter the conformation of the molecule and may explain why two seemingly similar structures may have different activities.

There are several features of the 10 compounds studied in Chapter 4 which may explain their toxicity profile. Positioning of a reactive groups within a structure may alter the toxicity of a molecule. For example, XR9006 is more toxic than XR9051 and this may be due to the fact that the para positioning of the tetrahydroisoquinoline group means that it is more likely to be exposed and therefore may be more prone to amide hydrolysis by proteases than in the case of XR9051 where the tetrahydroisoquinoline group is in the meta position compared to the benzylidene ring. The addition of alkyl groups to the molecule increases the lipophilicity. causing the compound to accumulate to high degree in the cell membrane. This may affect the integrity (i.e. fluidity and rigidity) of the membrane and increase the toxicity of the compound. The most toxic molecule XR9112 has an ethyl group rather than a hydrogen of methyl group attached to the nitrogen in the 5 position of the diketopiperazine ring. This may explains why XR9112 is more potent at lower doses and also why it is toxic at higher doses. The addition of alkyl groups also increases the occurrence of steric interactions within the molecule and therefore causes changes in molecular conformation. These conformational changes may have important implications in terms of toxicity and activity.

8.4 Xenova compounds as modifiers of MRP-mediated MDR

8.4.1 Introduction

All the compounds originally tested as modifiers of P-glycoprotein-mediated MDR were also tested for their activity as modifiers of MRP-mediated MDR. One of the initial test compounds, XR9089, showed activity as a modifier in MRP overexpressing cells. From this compound 10 more were synthesised and of these compounds XR9173 was the most efficient modifier of MRP-mediated MDR.

8.4.2 XR9173 is an effective modifier of P-glycoprotein- and MRP-mediated multidrug resistance

The data presented in Chapter 5 indicates that XR9173 is an efficient modifier of both P-glycoprotein- and MRP-mediated MDR. It is not surprising that the compound is an active modifier of P-glycoprotein-mediated MDR as the molecule has a tetrahydroisoquinoline group within its structure. The presence of this group within a molecule tends to increase its activity as a modifier of P-glycoprotein mediated MDR, possibly by increasing the positive charge of the molecule at physiological pH.

The possible reasons for the activity of XR9173 in MRP overexpressing cells are less straight forward. As MRP is thought to be identical to the GSH conjugate transporter (Jedlitschky *et al.*, 1994) and several groups have reported ATP-dependent transport of glutathione *S*-conjugates it is possible that XR9173 interacts with this transport system. Alternatively, it is possible that XR9173 interacts with the mechanism(s) involved in the transport of cationic drugs which may well be distinct from the GSH conjugate transporter.

8.4.3 The mechanism of action of XR9173 is unlikely to be competitive inhibition of the GSH conjugate transporter

As XR9173 is cationic it is unlikely that it exerts its activity in MRP overexpressing cells by itself being a substrate for the GSH conjugate/organic anion transporter thought to be identical to MRP. It does, however, modulate the accumulation and efflux of calcein, an anionic fluorescent dye. The data presented in Chapter 6 indicate that, like probenecid, XR9173 is able to inhibit the transport of daunorubicin and restore partially the calcein accumulation deficit in L23/R cells. It may, therefore, have a dual function being able to inhibit (partially) the organic anion transporter and also inhibit the, as yet undefined, mechanism responsible for causing a decreased intracellular accumulation of cationic cytotoxic drugs. These results provide evidence to support the theory that MRP may co-exist with another transporter which is able to transport cationic drugs across the membrane and that these transport are able in some way to regulate each others' activity.

8.5 The relationship between phosphorylation status and drug transport in multidrug resistant cells

The study presented in Chapter 7 demonstrates that inhibition of PKC with calphostin C decreased the phosphorylation of P-glycoprotein and increased the accumulation of daunorubicin and colchicine in the P-glycoprotein overexpressing MDR cells, CEM/VLB. The light-activated form of the PKC inhibitor calphostin C has a greater effect on the transport of cytotoxic drugs than the non-light-activated form. These results together with the observation than light-activated calphostin C is able to inhibit the ability of [^{3}H]azidopine to photolabel P-glycoprotein provide evidence that calphostin C causes an alteration in the drug binding capability of P-glycoprotein by dephosphorylating the protein. In addition TPA, a PKC activator, increases the phosphorylation of P-glycoprotein and reduces intracellular drug accumulation in the MDR cell line, CEM/VLB. The decrease in drug accumulation observed in the presence of TPA may indicate that either hyperphosphorylated P-

glycoprotein is a more efficient drug efflux pump or that TPA interferes in someway with the passive diffusion of drugs across the cell membrane. These results indicate that P-glycoprotein-mediated drug efflux may be modulated by phosphorylation and that this modulation may be a consequence, at least in the case of azidopine, of decreased binding. Non-light-activated calphostin C, however, significantly increases drug accumulation in the MDR cell line, CEM/VLB, without any effect on the phosphorylation status of P-glycoprotein. This may suggest that calphostin C is able to reverse MDR by a mechanism other than via inhibition of P-glycoprotein phosphorylation via irreversible inhibition of PKC. It is important, therefore, that the PKC activity and other actions of PKC inhibitors with respect to P-glycoprotein (i.e. ability to bind directly to P-glycoprotein) must be considered when hypothesising about their mechanism of action with respect to resistance modification.

8.6 Concluding remarks

When P-glycoprotein was first described it was thought to be a protein expressed only on drug resistant cells and hence potentially that elusive goal of chemotherapy the 'tumour-specific' target. When antibodies to P-glycoprotein became available, it became clear that the protein was widely expressed in normal tissues (Thiebaut *et al.*, 1978) and hence the ever-present dilemma of cancer chemotherapy- the narrow therapeutic ratio- was still present.

The clinical value of modulation of P-glycoprotein activity is still far from proven. Sikic (1993) argued that there is no proven case for the use of resistance modifiers in standard oncology practice. There is currently major clinical interest in lymphomas, leukaemias and myeloma. Modifiers are being combined with anthracyclines and *vinca* alkaloids. Large randomised trials are underway in both Europe and North America. Trials in drug resistant solid tumours are generally

more difficult to design and the results are more difficult to interpret. The limited trials carried out so far have been fairly uniformly negative.

For the future there are three major questions that remain unanswered. Firstly, what is the constitutive role of P-glycoprotein in many of the normal tissues in which it is expressed? Secondly, how is *MDR1* gene expression controlled on a transcriptional level? Finally, How do we measure P-glycoprotein expression in a sufficiently precise way to determine its clinical significance? It appears, therefore, that despite the fact that research into P-glycoprotein activity has been ongoing for over two decades, its clinical values are far from proven.

The other MDR protein discussed in this thesis, the multidrug resistance-associated protein (MRP) is still in the early stages of research compared to P-glycoprotein. As yet, there is not enough detailed information concerning its expression in cancers and normal tissues to determine its significance in MDR. More in vitro studies are required to elucidate more potent modifiers of MRP and their potential clinical values.

amphipathic

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